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(54) Title: ANALYSIS OF SOLUBLE FACTOR BIOLOGICAL MARKERS

(57) Abstract: Multiplexed bioassays of soluble factors by flow cytometry and associated methods and reagents are provided, including the use of trigger microparticles and detection microparticles to form complexes with soluble factors, and methods of detecting and quantitating the complexes in flow cytometer-based, multiplexed assays.

ANALYSIS OF SOLUBLE FACTOR BIOLOGICAL MARKERS

FIELD OF THE INVENTION

The present invention relates to multiplexed bioassays of soluble factors by flow cytometry and associated methods and reagents. In particular, the present invention relates to the use of trigger microparticles and detection microparticles to form complexes with soluble factors, and to methods of detecting and quantitating the complexes in flow cytometer-based, multiplexed assays.

BACKGROUND OF THE INVENTION

As a result of recent innovations in drug discovery, including genomics, combinatorial chemistry and high throughput screening, the number of drug candidates available for clinical testing exceeds the pharmaceutical industry's development and economic capacity. In 1998, the world's top pharmaceutical and biotechnology companies spent over $50 billion on research and development, more than one-third of which was spent directly on clinical development. As the result of a number of factors, including increased competition and pressure from managed care organizations and other payors, the pharmaceutical industry is seeking to improve the efficiency of clinical development and increase the safety and efficacy of new drugs brought to market.

The acceleration of drug discovery through innovations, therefore, has resulted in a clinical trials bottleneck. The numbers of therapeutic targets being identified and lead compounds being generated far exceed the capacity of pharmaceutical companies to conduct clinical trials as they are currently performed. Further, because the industry currently estimates that the average cost of developing a new drug is approximately $500 million, it is prohibitively expensive to develop all of the potential drug candidates.

The pharmaceutical industry is being forced to seek equivalent technological improvements in drug development. Clinical trials remain very expensive and risky, and often decision making is based on highly subjective analyses. As a result, it is frequently difficult to determine the patient population for whom a drug is most effective, the appropriate dose for a given drug and the potential for side effects associated with its use. Not only does this result in more failures in clinical development; it can also lead to approved products that may be inappropriately dosed, prescribed, or cause dangerous side effects. With an increasing number of drugs in their pipelines, pharmaceutical companies require technologies to identify objective

measurements of a drug candidate's safety and efficacy profile early in the drug development process.

Biological markers are characteristics that when measured or evaluated have a discrete relationship or correlation as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention. Pharmacologic responses to therapeutic intervention include, but are not limited to, response to the intervention generally, dose response to the intervention, side effect profiles of the intervention, and pharmacokinetic properties such as the rate of drug metabolism and the identity of the drug metabolites. Response may be correlated with either efficacious or adverse changes. Biological markers include patterns of cells or molecules that change in association with a pathological process and have diagnostic and/or prognostic value. Biological markers may include levels of cell populations and their associated molecules, levels of soluble factors, levels of other molecules, gene expression levels, genetic mutations, and clinical parameters that can be correlated with the presence and/or progression of disease. In contrast to such clinical endpoints as disease progression or recurrence, or to quality of life measures (which may be difficult to quantify and typically require an extended period of time to assess), biological markers may provide a more rapid and quantitative measurement of a drug's clinical profile. Single biological markers currently used in both clinical practice and drug development include cholesterol, prostate specific antigen ("PSA"), CD4 T-cells and viral RNA. Unlike the well known correlations between high cholesterol and heart disease, PSA and prostate cancer, and decreased CD4 positive T-cells and viral RNA in AIDS, the biological markers correlated with most other diseases have yet to be identified. As a result, although both government agencies and pharmaceutical companies are increasingly seeking development of biological markers for use in clinical trials, the use of biological markers in drug development has been limited to date.

United States Patent Application Serial No. 09/558,909, entitled "Biological Marker Identification System" (filed April 26, 2000), incorporated herein by reference, provides a biological marker identification system that is capable of sorting through the vast amounts of information needed to establish the correlation of the biological markers with disease, disease progression and response to therapy. This technology includes the instrumentation and assays required to measure hundreds to thousands of biological markers, an informatics system to allow such data to be easily accessed, software to correlate the patterns of markers with

clinical data, and the ability to utilize the resulting information in the drug development process.

In some preferred embodiments, the biological marker identification system uses microvolume laser scanning cytometry (MLSC). Applications of MLSC include its use to measure cell-type population changes and soluble factor changes during disease progression and during therapy. MLSC may be used to identify novel biological markers for multiple sclerosis and rheumatoid arthritis, for example. The use of MLSC for a number of applications is described in United States Patent Application Serial No. 09/558,094, entitled "Improved System for Microvolume Laser Scanning Cytometry" (filed April 26, 2000), incorporated herein by reference.

Flow cytometry is frequently used to assay cell populations by virtue of the presence (or absence) and level of particular biological markers on the surface of cells; however, flow cytometry (e.g., MLSC) can also be used in microparticle-based assays to quantitate soluble factors in biological fluids. Such assays typically comprise a microparticle (e.g., a liposome, oil droplet, or a bead) coupled to a primary antibody that is specific for the soluble factor, and a secondary fluorescently-labeled antibody that also recognizes the soluble factor. See Swartzman *et al.*, Anal. Biochem 271:143 (1999). The factor of interest is first bound to the microparticle and then fluorescently-labeled by the binding of the secondary antibody. In this system, the concentration of the soluble factor of interest may be determined by measuring the fluorescent signal associated with each microparticle in the sample. It is possible to perform multiple assays in the same sample volume by using a number of different microparticle types (each conjugated to a different primary antibody). In order to identify each microparticle type, microparticles can be prepared that are distinguishable based on, for example, particle size or the fluorescence wavelength. "Color coding" of microparticles can be accomplished using dyes in the interior of the microparticle (e.g., lipophilic dyes for polystyrene beads or hydrophilic dyes for liposomes). Alternatively, the dye can be located externally; for example, adsorbed, covalently attached, or otherwise coupled (either directly or indirectly) to the surface of the microparticle.

Thus, biological marker analysis may be performed by using flow cytometry to simultaneously identify the type (or "flavor") of microparticle (and hence, if known, the soluble factor to which it binds) and quantitating the amount or concentration of that factor by measuring the fluorescence signal present at that microparticle resulting from binding of the secondary antibody. In alternative approaches, the microparticles all have the same attributes,

but are coupled with distinct primary reagents (e.g., antibodies), while the secondary reagents are labeled with a different fluorophores. Under these approaches, measurement of the signal from the secondary reagent reveals both the identity and the concentration of the bound soluble factor.

Flow cytometry systems can be configured to measure the fluorescence signals associated with differentiable microparticles. For example, MLSC instruments can be configured to distinguish microparticles based on a number of predetermined physical characteristics. As a result, multiple biological markers can be accurately quantitated simultaneously, even in the presence of a high fluorescence background. This means that soluble biological markers can be assayed in whole, or minimally processed, biological fluids simply by adding the microparticles and secondary antibodies, without requiring any washing out of the fluorescently-labeled secondary antibody. As a result, the assays can be performed quickly and with little (if any) processing. Furthermore, flow cytometry systems allow assays for both soluble factors and cell-expressed markers to be performed simultaneously in the same assay.

Methods for using microparticles to perform multiplexed cytometric assays of soluble factors are known in the art. A system for performing multiplexed assays involving multiple bead populations, for example, is described in United States Patent No. 5,891,738, entitled "Biospecific Multiparameter Assay Method." In that system, each population of microparticles is labeled with a different primary reagent capable of binding to a factor of interest. Each population of beads is also coupled to a unique fluorescent molecule (D_k). Thus, for a four-factor assay, four populations of beads are used, each with a different primary reagent, and each coupled to one of four different D molecules (i.e., D_1, D_2, D_3, D_4). The beads are pooled and incubated with the assay sample in the presence of secondary reagents that also bind to the particular factors of interest, but at sites distinct from the primary reagent. As a result, each bead binds to a particular soluble factor, and the bead-bound factor also binds to its cognate secondary reagent. The secondary reagents are all labeled with a common fluorescent molecule (F) that is distinct from the fluorescent molecules that are coupled directly to the beads (D_1, D_2, D_3, D_4,). After incubating the beads and the secondary reagents with the assay sample, the beads are analyzed by a confocal fluorescence detection system. For each bead, the system determines the identity of the factor that has bound to the bead by determining the identity of the D molecule by virtue of its emission characteristics. The

system then measures the fluorescent signal from the F molecule at each bead to quantitate the amount of factor that has bound to each bead.

One drawback to this system is that the ability to perform multiplexed assays is limited by the number of suitable D molecules. To allow unambiguous determination of the identity of a particular bead, the D molecules must be spectroscopically resolvable from one another. Moreover, the D molecules also have to be spectroscopically resolvable from the F molecules so that the factor bound to each bead can be accurately quantitated. Finally, all of the D molecules have to have the same excitation requirements as the F molecule, to allow single-wavelength excitation of the sample. These limitations in the characteristics of the D and F molecules reduce the number of potential candidate molecules, and hence the degree of multiplexing. Another drawback of this system is that the fluorescent signal for quantitation is limited by the number of F molecules that can be conjugated to a single secondary reagent. For example, in most cases the secondary reagent will be an antibody molecule, and only a limited number of F molecules can be conjugated to a single antibody without interfering with the binding properties of the antibody. Similarly, the factors can bind only a limited number of secondary reagents. Both of these limitations adversely affect the sensitivity of the system, making factors present at low levels in the sample difficult or impossible to detect.

In WO 97/14028, entitled "Multiplexed Analysis of Clinical Specimens Apparatus and Method," a system for multiplexed flow cytometry is described in which a number of populations of beads are synthesized, each population being coupled to an antibody against a factor to be measured. The bead populations have unique physical characteristics that can be detected by means of a flow cytometer. For example, the beads in different populations can have different forward light scatter values or different side light scatter values. Alternatively, each population of beads can be labeled with one or two fluorophores, so that the fluorescence intensity, or the fluorescence emission ratio, of different bead populations (measured at appropriate wavelengths) is distinct. These parameters can be combined to yield beads that have up to four distinct characteristics, thereby increasing the potential degree of multiplexing. The beads are incubated with the assay sample and contacted with secondary antibodies that recognize the factors of interest. Each secondary antibody binds to a factor at a site distinct from the primary antibody, and all the secondary antibodies are labeled with a common fluorophore. The beads are then analyzed using flow cytometry. As each bead passes through the flow cytometer, its identity is determined by virtue of its physical properties, and the amount of factor bound is quantitated by measuring the fluorescence signal from the secondary

antibody. Again, the applicability of this approach is hampered by the limited number of physical parameters that can be detected simultaneously. Moreover, because only a limited number of fluorophore molecules can be conjugated to each secondary antibody, this system suffers from the same sensitivity limitations described above; factors can bind only a limited number of secondary reagents which, in turn, can only be labeled with a limited number of fluorescent moieties.

The use of microparticles of different sizes and colors, in combination with secondary antibodies with fluorescent labels, allows limited multiplexed analysis of biological factors to be performed by flow cytometry; however, it would be desirable to have even further diversity in these reagents. The objective of the present invention is to provide improved methods and reagents for the multiplexed analysis of soluble factors that address the problems mentioned above.

SUMMARY OF THE INVENTION

The present invention provides methods and reagents for the multiplexed analysis of soluble factors using flow cytometry. For each soluble factor to be analyzed, the invention uses at least two different sets of microparticles, preferably beads. The first set of microparticles, referred to as "trigger microparticles," is labeled with a primary reagent that binds to a soluble factor to be detected. The second set of microparticles, termed "detection microparticles," is labeled with a secondary reagent that binds to the same soluble factor as the first reagent such that the soluble factor can be bound simultaneously by the detection microparticles and the trigger microparticles. In some embodiments, for example, the detection microparticle reagent may bind directly to the factor (e.g., at a site other than the site bound by the trigger microparticle reagent). In alternative embodiments, the soluble factor may be chemically modified (e.g., by the addition of a biotin group via a biotinylating reagent) and the detection microparticles coupled to a reagent capable of binding to the modifying group. Thus, if the modifying group is biotin, then the second reagent can be streptavidin. In either case, the simultaneous binding of the trigger and the detection microparticles brings the microparticles into close proximity. High levels of multiplexing can be achieved by using trigger and detection microparticles with variable physical properties such as size, shape, fluorescence label identity, fluorescence emission intensity, fluorescence emission ratio, and so on. Each soluble factor in the assay will have a unique detectable trigger-detection microparticle composite signal in flow cytometry.

The methods of the present invention can be used to perform flow cytometry-based analysis of soluble factors in biological fluids. In addition, they can be used in a number of other multiplexed assays, such as analysis of PCR products in order to rapidly obtain genotype information, or to detect nucleic acids associated with pathogens.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 illustrates schematically one embodiment of the invention for the analysis of three soluble factors, factor *a* (black hexagon) factor *b* (black triangle) and factor *c* (black square) in a multiplexed assay. FIGURE 1A depicts three populations of trigger microparticles, T*a*, T*b*, and T*c*, each labeled with a primary antibody (antibodies are depicted as "Y" shapes). Trigger microparticle T*a* is bound to an antibody that recognizes the soluble factor *a*; trigger microparticle T*b* is bound to an antibody that recognizes soluble factor *b*; and trigger microparticle T*c* is bound to an antibody that recognizes soluble factor *c*. In this embodiment, the different populations of trigger microparticles are of different sizes, which will allow them to be separately identified by flow cytometry. FIGURE 1B illustrates binding of the soluble factors to the corresponding trigger microparticles. In FIGURE 1C, the binding of detection microparticles to the soluble factors is depicted. Each detection microparticle is labeled with a secondary antibody that binds to a soluble factor at a site distinct from the site at which the primary antibodies of the trigger microparticles bind. Detection microparticle D*a* binds to soluble factor *a*; detection microparticle D*b* binds to soluble factor *b*; and detection microparticle D*c* binds to soluble factor *c*. In the presence of their cognate soluble factors, the detection microparticles and the trigger microparticles form complexes (as shown). If the detection microparticles are fluorescently labeled, then the levels of soluble factor *a*, *b*, and *c* can be determined by (1) identifying each trigger microparticle in the assay as trigger microparticle T*a*, T*b*, or T*c*; and then (2) measuring the fluorescent signal contributed by the detection microparticle associated with each identified trigger microparticle. Because the identity of the soluble factor is specified by the identity of the trigger microparticle to which it binds, all the detection microparticles can be (but do not have to be) labeled with the same fluorophore.

FIGURE 2 depicts schematically an embodiment of the invention in which the soluble factor of interest (hatched circle) binds to (1) a primary antibody on the surface of the trigger microparticle and (2) a biotinylated secondary antibody. Commonly, the primary antibody is specific for the factor of interest. In some applications, it may also be important for the

secondary antibody to be specific (e.g., if similar analytes are being distinguished); however, in many applications, specificity is not required. A detection microparticle conjugated to streptavidin will bind to the biotinylated secondary antibody, thereby forming a trigger:detection complex. As shown the detection microparticle is fluorescently-labeled (fluorophores depicted by "F"), allowing quantitation of the factor bound to trigger microparticles in the assay.

FIGURE 3 illustrates schematically a multiplexed assay for soluble factor *a* (black hexagons), factor *b* (black triangles) and factor *c* (black square) using biotinylated secondary antibodies and streptavidin-labeled detection microparticles. In FIGURE 3A, trigger microparticles T*a*, T*b* and T*c* labeled with primary antibodies specific for soluble factor *a*, factor *b*, and factor *c*, respectively, are added to the sample to be assayed and specific binding is allowed to take place. The result is shown in FIGURE 3B. In FIGURE 3C, biotinylated secondary antibodies specific for sites on soluble factors *a*, *b*, and *c* that are distinct from those recognized by the primary antibodies are added to the assay mixture (biotin is depicted by "B"). In FIGURE 3D, fluorescently labeled detection microparticles (hatched circles) conjugated to streptavidin are added to the assay (streptavidin is depicted by "S"). The detection microparticles bind to secondary antibodies via the biotin-streptavidin interaction, thereby bringing detection microparticles together with the trigger microparticles. A single population of fluorescently-labeled detection microparticles can function as a generic detection reagent in this illustrative example.

FIGURE 4 depicts schematically an embodiment of the invention in which the soluble factor of interest competes for binding to the trigger microparticle with exogenously-supplied biotinylated soluble factor. A trigger microparticle coupled to an antibody that will bind the soluble factor is introduced to the sample. Exogenous biotinylated soluble factor introduced to the sample and endogenous unmodified soluble factor will compete for binding to the trigger microparticles. The (exogenous) biotinylated soluble factor can bind to a streptavidin coupled microparticle that is also fluorescently labeled. The complex formed by the trigger and the detection microparticles both binding to the biotinylated the soluble factor can be determined by MLSC. By comparing the binding data with a standard curve of the competition between biotinylated and unmodified soluble factor, it is possible to determine the concentration of the (endogenous) unmodified soluble factor present in the sample.

FIGURE 5 depicts schematically a competitive microparticle agglutination assay using the methods of the present invention. The soluble factor to be measured (D) is coupled to a

dye-conjugated microparticles (open circle) by any means known in the art (e.g., via a linker or an antibody). A high affinity antibody (depicted as "Y" shapes) that recognizes factor D is coupled to a second dye-conjugated microparticle (shaded circle). Preferably, the different types of microparticles are conjugated to different dyes. Both microparticle types are introduced to the sample to be assayed. In a sample that does not contain factor D, agglutination will be observed because the anti-factor D antibodies on the first type of particle will bind to the factor D bound to the second type of particle. This process is illustrated in left-hand pathway in the figure. In a sample that does contain factor D, agglutination will be inhibited or reduced as a function of the concentration of free factor D in the sample. This is because the anti-factor D antibodies on the first type of particle will bind to the free factor D in the sample. Accordingly, the degree of agglutination inhibition can be used to quantify the amount of free factor D in the sample. This process is illustrated in the right-hand pathway in FIGURE 5.

FIGURE 6 depicts schematically a non-competitive microparticle agglutination assay using the methods of the present invention. Such assays are appropriate when the soluble factor to be quantitated has two or more non-overlapping epitopes. The soluble factor to be measured is shown as having non-overlapping epitopes, A and B. Two sets of dye-conjugated microparticles are prepared. The first (open circle) is labeled with anti-A antibodies (depicted as thin "Y" shapes) and second (shaded circle) is labeled with anti-B antibodies (depicted as thick "Y" shapes). Preferably, the different types of microparticles are conjugated to different dyes. When these particles are introduced to a sample containing free soluble factor of interest, mixed agglutinates will be formed as the particle-coupled antibodies bind to the soluble factor.

DETAILED WRITTEN DESCRIPTION OF THE INVENTION

The present invention is directed to methods and reagents useful for the analysis of soluble factors. Such factors may function as biological markers of diseases or medical conditions, or response to therapy. In particular, the invention is directed to a system for soluble factor analysis using flow cytometry. To aid in the understanding of the invention, the following definitions are provided:

As used herein the term "biological marker" or "marker" or "biomarker" means a characteristic that is measured or evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention.

Pharmacologic responses to therapeutic intervention include, but are not limited to, response to the intervention generally, dose response to the intervention, side effect profiles of the intervention, and pharmacokinetic properties. Response may be correlated with either efficacious or adverse or toxic changes. Biological markers include patterns or ensembles of cells or molecules that change in association with a pathological process and have diagnostic and/or prognostic value.

Biological markers include, but are not limited to, cell population counts, and levels of associated molecules, levels of soluble factors, levels of other molecules, gene expression levels, genetic mutations, and clinical parameters that can be correlated with normal biologic processes or with the presence and progression of disease and response to therapy. Biological markers can also identify subsets of patients with particular diseases that respond differently to various therapies. The present invention is directed towards methods of quantitating biological markers that are present in biological fluids as soluble factors. As mentioned above, certain biological markers (such as cholesterol, PSA, CD4 T-cells, and viral RNA) are currently used in both clinical practice and drug development; however, biological markers correlated with most diseases have yet to be identified.

As a non-limiting example, biological markers include those characteristics often thought of as having discrete relationships with normal biological status, a disease or medical condition. For example, high cholesterol correlates with an increased risk of heart disease, elevated PSA levels correlate with increased risk of prostate cancer, reduced CD4 T-cells and increased viral RNA correlate with the presence and progression of AIDS. It is already clear that using more than one biomarker improves predictive value. For example, an elevated cholesterol level together with a subnormal low-density lipoprotein cholesterol level is better correlated with risk of coronary artery disease than is either marker alone. It is likely that useful predictors for a variety of diseases or medical conditions will require combinations of biomarkers. For example, it could be discovered that lowered levels of one or more specific cell-surface antigens on specific cell type(s), when found in conjunction with elevated levels of one or more soluble proteins, such as cytokines, are indicative of a particular auto-immune disease. Therefore, for the purposes of this invention, a biological marker may refer to a pattern of, or relationship between, a number of characteristics.

As used herein the term "biological fluid" means any biological substance, including but not limited to, blood (including whole blood, leukocytes prepared by lysis of red blood cells, peripheral blood mononuclear cells, plasma, and serum), sputum, saliva, urine, semen,

cerebrospinal fluid, bronchio-alveolar lavage fluid, sweat, feces, synovial fluid, lymphatic fluid, tears, and macerated or homogenized tissue obtained from any organism. Biological fluid typically contains cells and their associated molecules, soluble factors, small molecules and other substances. Blood is the preferred biological fluid for use with respect to this invention for a number of reasons. First, it is readily available and can be drawn at multiple times. Blood cells are replenished, in part, from progenitors in bone marrow over time. Blood is responsive to antigenic challenges and has a memory of antigenic challenges. Blood re-circulates and potentially reports on changes throughout the body. Blood contains numerous cell populations, each of which has surface molecules, internal molecules, and secreted molecules that may vary in individual cells. Blood also contains soluble factors that are both self, such as cytokines, antibodies, acute phase proteins, etc., and foreign, such as drugs or other chemicals and products of infectious agents.

As used herein the term "soluble factor" means any soluble molecule that is found in a biological fluid, typically blood. Soluble factors include, but are not limited to, soluble proteins, peptides, carbohydrates, lipids, lipoproteins, steroids, other small molecules, and complexes of any of the preceding components; for example, cytokines and soluble receptor, antibodies and antigens, and drugs complexed to another entity. Soluble factors can be classified as "self," such as cytokines, antibodies, acute-phase proteins, etc., or "foreign," such as chemicals and products of infectious diseases. Soluble factors may be classified as "intrinsic" (i.e., those produced by the individual) or "extrinsic" (i.e., those not produced by the individual, such as a virus, drug or environmental toxin). Soluble factors can be small molecule compounds such as prostaglandins, vitamins, metabolites (such as sugars, amino acids, etc.), drugs and drug metabolites. The soluble factor can be polyvalent or polyepitopic (having more than one binding site for a ligand) or monovalent. Polyvalent soluble factors include, but are not limited to, polypeptides, proteins, DNA and RNA strands in their different forms (e.g., *m*-, *r*-, *t*- as well as duplexes, heteroduplexes, triplestrands), polysaccharides or combinations, chromosomes, genes, cells, cell membranes or cell parts (e.g., nucleus, mitochondria), bacteria, viruses, or other microorganisms. Monovalent soluble factors contemplated by the invention include, but are not limited to, natural metabolites, hormones, vitamins, drugs, drug metabolites, pollutants, and pesticides. For polyvalent soluble factors, the invention contemplates the use of sandwich assays well known in the art. For monovalent soluble factors, the invention contemplates the use of competition assays.

As used herein the term "small molecule" or "organic molecule" or "small organic molecule" means a soluble factor or cell-associated factor having a molecular weight in the range of 2 to 2000 Daltons. Small molecules can include, but are not limited to, peptides, polypeptides, nucleotides, oligonucleotides, polynucleotides, prostaglandins, vitamins, metabolites (such as sugars, amino acids, etc.), drugs and drug metabolites. In one important embodiment, the MLSC system is used to measure changes in the concentration of drugs and drug metabolites in biological fluids in tandem with other biological markers during a treatment regime.

As used herein, the terms "flow cytometer" and "flow cytometry" is used to refer to the instruments and optical techniques used to analyze particles in a fluid suspension by virtue of the particles characteristics (e.g., optical or physical characteristics). Typically, the particles are caused to flow past a focused beam of light (e.g., laser light) that illuminates the particles and optical detectors measure characteristics of the light resulting from the interaction (e.g., scatter, fluorescence). See Shapiro, "Practical Flow Cytometry," 3rd Ed., (Alan R. Liss, Inc. 1995). Thus, flow cytometry includes methods for detecting the presence of a component in a small volume of a sample using a fluorescently labeled detection molecule and subjecting the sample to optical scanning where the fluorescence emission is recorded. Included within the definition is "Microvolume Laser Scanning Cytometry" or "MLSC" or "MLSC systems." The MLSC system used in some preferred embodiments of the instant invention has several key features that distinguish it from other technologies: (a) only small amounts of blood (5-50 μl) are required for many assays; (b) absolute cell counts (cells/ μl) are obtained; and, (c) the assay can be executed either directly on whole blood or on purified white blood cells. Laser scanning cytometry with microvolume capillaries provides a powerful method for monitoring fluorescently labeled cells, microparticles, and soluble factors from samples (e.g., whole blood, processed blood, and other fluids, including biological fluids).

MLSC technology is described, for example, in United States Patent Nos. 5,547,849 and 5,556,764 and, in Dietz *et al.*, Cytometry 23:177-186 (1996), each of which is specifically incorporated herein by reference in its entirety. A number of enhancements of the basic MLSC technology have been made, and are specifically contemplated by the present invention. See, e.g., United States Patent Application No. 09/378,259, entitled "Novel Optical Architectures for Microvolume Laser-Scanning Cytometers" (filed August 20, 1999), specifically incorporated herein by reference in its entirety.

The IMAGN® 2000 system, commercially available from Biometric Imaging Inc., is an example of a MLSC system. Preferably, a helium-neon laser with a wavelength of 633 nm is used. This wavelength avoids problems associated with the autofluorescence of biological materials. The power of the laser is increased from 3 to 17 mW. Higher laser power has two potential advantages, increased sensitivity and increased scanning speed. Also included within the definition is fluorometric microvolume assay technology (FMAT) designed to perform screening assays on cells or beads, including fluorescent ligand receptor binding assays, fluorescent immunoassays, and cytotoxicity and apoptosis assays. The FMAT™ 8100 HTS System is commercially available from PE Biosystems (Foster City, CA).

As used herein the term "dye" refers to a means a molecule capable of emitting light in response to appropriate stimulation. In preferred embodiments, the dye is a molecule which, following absorption of light at wavelength of 250 to 110 nm emits light by fluorescence, or phosphorescence, or transfers its excitation energy to an acceptor molecule which thereupon emits light by fluorescence or phosphorescence. Energy transfer can occur through, for example, fluorescence resonance energy transfer (FRET) or a Dexter-type exchange mechanism. See, Dexter, J. Chem. Phys. 21:836 (1953), incorporated herein by reference in its entirety. The emission quantum yield of the dyes should be at least 0.1 and the extinction coefficient of absorption should be greater than $5000M^{-1}$ cm^{-1}. A significant number of dyes are known in the art. Suitable dyes include xanthenes, such as fluorescein and rhodamine, coumarins, such as umbelliferone, aromatic amines, such as dansyl, squarate dyes, benzofurans and polyaromatic hydrocarbons, such as anthracenes, naphthacenes, and porphyrins, cyanines, naphthalocyanines or lanthanide chelates (Eu, Tb, Sm) where both lifetime and wavelength are measured. The lanthanide chelates are particularly attractive for some applications because of their sharp band of luminescence.

Light emission can occur through phosphorescence or, preferably, through fluorescence or chemiluminescence. The terms "fluorophore," "fluorescent label," and "fluorescent group" are used interchangeably throughout this application to refer to fluorescent dyes. The dye is typically directly linked to a detection microparticle in the present invention, although indirect linkage is also encompassed herein. A significant number of dyes are known in the art. In certain preferred embodiments, fluorophores are used which can be excited in the red region (> 600 nm) of the spectrum. Two red fluorophores, Cy5 and Cy5.5, are commonly used. They have emission peaks of 665 and 695 nanometers, respectively, and can be readily coupled to antibodies. Both can be excited at 633 nm with a helium-neon laser. Sets of three

red fluorophores that may be used include, Cy5, Cy5.5 and Cy 7 or Cy5, Cy5.5 and Cy 7-APC. Such red fluorophores are preferred, but not required, when the assays of the invention are conducted in whole blood. If the assay includes a washing step to remove unbound blood constituents, then any fluorophore that absorbs light of wavelength between 240 nm and 1200 nm may be used. The present invention also includes dyes suitable for use in MLSC, and methods for their synthesis. See e.g., United States Patent Application No. 09/612,331, entitled "Bridged Fluorescent Dyes, Their Preparation and Their Use in Assays" (filed July 7, 2000), incorporated herein by reference. The fluorescence quantum yield of dyes is often negatively affected by environmental factors such as solvent polarity, pH, and interactions with proteins or DNA. Thus, in some embodiments, the dyes may advantageously be encapsulated into a hydrophobic medium such as a latex particle. This will not only alleviate these environmental problems but also increase the overall fluorescent brightness of the dye.

As used herein, the term "microparticle" means any macromolecular assembly to which reagents may be coupled. In some preferred embodiments, the microparticles of the present invention are beads comprised of latex or polystyrene; however, the invention also contemplates the use of other types of microparticles, including but not limited to, liposomes, emulsion droplets, dendrimers and Quantum Dots™ particles ("Q-dots™") (Quantum Dot Corp., Palo Alto, CA). In other preferred embodiments, the microparticles used are Nanobar Codes™ identification tags. Nanobar Codes™ tags are described in United States Patent Application No. 09/677,198, entitled "Colloid Rod Particles as Nanobar Codes" (filed October 2, 2000), incorporated herein by reference. In one embodiment, Nanobar Codes™ tags are cylindrical in shape, with segmented composition that varies along the length of the rod. The rods can be prepared, for example, by sequential electrochemical reduction of metal ions (such as Pt^{2+} and Au^{+}, among other) in porous alumina membranes and then release into solution following membrane dissolution. Because the width of the segments can be adjusted by controlling the amount of current passed in each electroplating step, the rod resembles a "bar code" on the nanometer scale, with each segment width (and identity) programmable in advance. While the width of the rods and the segment lengths are of nanometer dimensions, the overall length is such that it can be visualized directly in an optical microscope, exploiting the differential reflectivity of the metal components. The flow cytometry systems referred to above can be readily adapted to perform the reflectivity measurements required for detecting Nanobar Codes™ tags. If the rods consist of 9 equal segments, and there are 4 different metals (each with a different reflectivity) from which the segments may be composed, then

there are 4^9 (>260,000) unique types of bar code metal nanoparticles that could be prepared. Each of these Nanobar Codes™ tags can be conjugated to a different reagent (e.g., an antibody) and used in multiplexed assays.

In other embodiments, the microparticles or nanoparticles are SERS-active composite nanoparticles (SACNs). See J. Raman Spectroscopy 30, 785-793 (1995), specifically incorporated herein by reference in its entirety. SACNs typically comprise a metal nanoparticle core upon which is absorbed one or more species of Raman-active molecule. The core is encapsulated by a network of polymer or, preferably, glass. The resulting SACN retains the robust Raman signature of the coated metal core, but unlike naked metal nanoparticles, is resistant to aggregation and decomposition of the Raman species. Molecules can be conjugated to the exterior surface of the SACN via standard techniques. Because literally hundreds of different Raman activities may be resolved from one another in a single Raman spectrum, SACNs provide an enormous pool of spectrally diverse (but otherwise uniform) microparticles. The MLSC systems referred to above can be readily adapted to measure Raman spectra.

In one series of embodiments, the present invention provides a system for the detection and quantitation of a biological marker comprising a soluble factor in which the reagents comprise: (a) a trigger microparticle coupled to a primary reagent capable of binding to the soluble factor; and (b) a secondary reagent associated with a dye-conjugated detection microparticle, and capable of binding to the same soluble factor bound by the trigger microparticle reagent.

A flow cytometry instrument scans the assay sample in the presence of complexes (formed by the trigger microparticle, the soluble factor, and the detection microparticle), and acquires images at the appropriate wavelengths for capturing the light emitted by the dye(s). In the resulting output image, the flow cytometry image analysis software identifies each trigger microparticle; the dye signal contributed by the detection microparticle is then measured at each trigger microparticle in the image file.

Preferred embodiments of the invention use beads as the trigger and detection microparticles, and fluorophores as the dyes. The beads can be comprised of, for example, latex or polystyrene. Alternatively, the microparticles can comprise an emulsion droplet, a liposome, Nanobar Codes™ identification tags, SERS-active composite nanoparticles (SACNs), or Q-dots™ particles. The invention contemplates the use of any microparticle to which primary and secondary reagents can be coupled. Moreover, a different type of

microparticle may be used for the trigger microparticle than is used for the detection microparticle. For example, the trigger microparticle may be a Nanobar Codes™ tag while the detection microparticle may be a fluorescently labeled bead. In addition, a trigger particle could be a combination of individual particles. For example, two or more Q-dots particles could be crosslinked or encapsulated in a polymeric matrix to produce a trigger particle having a unique spectral profile corresponding to the sum of the spectral characteristics of the individual Q-Dots.

In preferred embodiments of the invention, multiple soluble factors are detected and quantitated simultaneously. A population of trigger microparticles is prepared for each soluble factor to be measured. The trigger microparticles in each population are labeled by any technique known in the art with a primary reagent that is capable of binding to the soluble factor. The primary reagent can be a receptor, a lectin, a DNA single strand or any other binding protein or molecule having a high affinity for the soluble factor. In some preferred embodiments, the primary reagent is an antibody molecule, or fragment thereof, that is capable of binding with high affinity and specificity to the marker. Although many preferred embodiments of the present invention use antibodies to detect biological markers, any other detection molecule capable of binding specifically to a particular biological marker is contemplated. For example, various types of receptor molecules can be detected through their interaction with a fluorescently-labeled cognate ligand and oligosaccharides can be detected following binding of a labeled lectin.

Thus, for three markers, three trigger microparticle populations could be synthesized, each labeled with a different primary reagent (See FIGURE 3). Antibodies can be attached to trigger microparticles by a number of methods well known in the art. For example, the antibody can be physically adsorbed onto the microparticle (see Example 3), it can be covalently linked to the microparticle, or it can be biotinylated and attached to streptavidin-coated trigger microparticles. Methods for the manipulation of antibodies in this way are familiar to those of skill in the art. For example, the trigger particle could be labeled with an anti-murine immunoglobulin (often referred to as catching antibody) before incubating with anti-soluble factor murine capture antibody (primary reagent). In addition to animal specific antibodies and biotin/avidin, other well-known mechanisms (such as digoxigenin/antidigoxigenin or complementary DNA and/or RNA strands) can be used to couple a trigger particle to a primary reagent. Of course the same systems can be used to attach the secondary reagent to the detection particle.

More generally, the trigger microparticle can be used in the same way the solid phase is used in an enzyme-linked immunosorbent assay (ELISA) or fluorescent-linked immunosorbent assay (FLISA). Thus, direct, indirect, sandwich, competition assay formats can be achieved using the trigger and detection particles of the present invention.

In order to discriminate between the different trigger microparticles during a multiplexed flow cytometry assay, each population may be characterized by unique attributes or characteristics (including without limitation size, shape, spectral signature, segmentation number or pattern). Being able to recognize each trigger microparticle, the flow cytometry system can provide information that will reveal the identity of the bound soluble factor. For example, each different populations of trigger microparticles can be labeled with a unique dye, preferably a fluorophore. If it is known what primary reagent is present on the surface of the trigger particle (e.g., antibody specific for factor *a*), then identifying the trigger particle will reveal the identity of the bound factor (e.g., factor *a*). In other embodiments, the trigger microparticles may be labeled with the same dye, but in a unique concentration for each different population. In still further embodiments, each population can be labeled with a unique profile of two or more dyes; in such cases, each different population of trigger microparticles will have its own unique emission ratio.

It will be understood that there are many such parameters that can be used to identify microparticles with a unique attributes. Indeed, any parameter that can be recognized by flow cytometry can form the basis for differentiating populations of trigger microparticles and is therefore contemplated by the present invention. Additionally, it will be recognized that combinations of the characteristics described above can further increase the complexity of the possible assays. To illustrate: A system that uses 10 different trigger microparticle sizes, each labeled with one of 10 different fluorophores will potentially provide 100 different trigger microparticle populations. If each fluorophore can be present at one of 10 different concentrations, 1,000 different populations of trigger microparticles will be possible. In each case, the population comprises microparticles that have a unique combination of attributes (e.g., size, fluorescence emission spectrum, and fluorescence emission intensity). The image analysis algorithms of the MLSC system can be configured to recognize particles with such physical characteristics. Each different population of trigger microparticles may be labeled with a specific primary reagent. Thus, in a system that allows 1,000 differentiable types of trigger particles, 1,000 different primary reagents (probes) could be used in a single multiplexed assay.

Some preferred embodiments use trigger microparticle size and/or dye signal to discriminate between microparticle populations; however, it will be appreciated that there are many other ways of preparing microparticles that would allow them to be differentiated from one another. For example, trigger microparticles can be synthesized that have different absorbance spectra, infrared spectra, reflectivity profiles, or time-resolved fluorescence profiles. In one especially preferred embodiment, the trigger microparticles comprise Nanobar Codes™™ identification tags where the reflective pattern on the tag can specify the identity of the soluble factor bound thereto. In other embodiments, the trigger microparticles could be surface-enhanced Raman spectroscopy (SERS)-active composite nanoparticles, each having a unique Raman spectrum. Such nanoparticles are described in Vo-Dinh, Trends in Analytical Chemistry, 17:557-582 (1998), incorporated herein by reference. In particular, the MLSC systems referred to in this application can be readily configured to obtain Raman spectra.

The trigger microparticles described above are contacted with the biological sample being assayed so that the primary reagent may bind soluble factors that are present in the sample. The trigger microparticle and the sample are preferably contacted under conditions (e.g., temperature, pH) that allow for binding between the primary reagent and the soluble factor. In addition, care should be taken to avoid the use of buffers, chelation agents, or other components that would prevent binding or would cross-react with the factor.

In order to detect binding of a soluble factor to a trigger microparticle, detection particles are used. In some embodiments, the detection particles are also microparticles coupled to a secondary reagent that binds to the soluble factor bound by the primary reagent on the trigger microparticles. In some embodiments, the secondary reagent simply binds to a different region of the soluble factor than is bound by the primary reagent. For example, the primary and secondary reagents can both be antibodies that bind to different epitopes present on the same soluble factor. In other embodiments, the soluble factor may be modified, and the secondary reagent binds to the modified portion or moiety of the factor of interest. As a result of the interactions between the primary and secondary reagent with the soluble factor, the trigger microparticles and the detection microparticles are brought into close proximity to one another. In preferred embodiments, the detection microparticles are smaller in size than the trigger microparticles to allow the clustering of multiple detection microparticles around a single trigger microparticle. This clustering enhances the sensitivity of the assay by increasing the amount of detection microparticle-contributed dye signal that can be associated with a single trigger microparticle.

In preferred embodiments, each population of detection microparticles is fluorescently labeled. Because the attributes of the trigger microparticle yield information about the identity of the soluble factor that has bound to a particular trigger microparticle, all populations of detection microparticle can be labeled with the same fluorophore. In this case, the flow cytometry (e.g., MLSC) system software determines the identity of the soluble factor that has bound to each trigger microparticle by measuring the physical characteristics of the trigger microparticle. The software then measures the fluorescent signal contributed by detection microparticle thereby yielding quantitative data concerning the amount of soluble factor bound to each trigger microparticle. In this way, MLSC analysis produces data on the concentration and identity of each soluble factor obtained from the sample.

For example, to determine the concentration of soluble factors *a*, *b*, and *c*, three uniquely-sized populations of trigger microparticles, T*a*, T*b* and T*c*, and three cognate populations of fluorescein-labeled detection microparticles, D*a*, D*b*, and D*c*, may be used. Soluble factor *a* will bind to T*a*, soluble factor *b* will bind to T*b*, and soluble factor *c* will bind to T*c*. Detection microparticle D*a* will then bind to soluble factor *a* (bound to trigger microparticle T*a*). As a result, the fluorescein signal of D*a* is brought to the vicinity of T*a*. Thus, the magnitude of the fluorescein signal at each T*a* microparticle is proportional to the number of D*a* microparticles that have become complexed to the T*a* microparticle; which, in turn, is proportional to the amount of soluble factor *a* that has bound to T*a*. The MLSC system determines the concentration of soluble factor *a* in the sample by first identifying all the T*a* microparticles in the raw image by virtue of their unique size, and then measuring the magnitude of the fluorescein signal at each identified T*a* microparticle. The accumulated observations for all the T*a* microparticles in the sample yields information about the concentration of soluble factor *a* in the sample. The same approach is followed for the quantitation of soluble factors *b* and *c*. FIGURE 1 shows this process schematically.

The secondary reagents can be associated with the detection microparticles by any method known in the art for the attachment of molecules to solid surfaces. For example, if the secondary reagent is an antibody, the detection microparticles can be labeled via a covalent linkage, or through physical adsorption. In some preferred embodiments, the secondary antibodies are coupled to detection microparticles via the biotin-streptavidin interaction. Specifically, the secondary antibodies are labeled with biotin, and the detection microparticles are labeled with streptavidin. The binding of the secondary antibodies to the detection microparticles can take place either before or after the secondary antibody has bound to the

soluble factor. Complexes between trigger and detection microparticles are formed when a soluble factor binds to the trigger microparticle (via interaction with the primary antibody) and also to the biotin-labeled secondary antibody, which in turn binds to the streptavidin-linked detection microparticle. FIGURE 2 illustrates these interactions schematically. This embodiment is especially preferred because it does not involve the preparation of separate populations of detection microparticles prior to the assay. Instead, a multiplexed assay can be performed by adding a single population of streptavidin-labeled detection microparticles (labeled with a dye such as a fluorophore), along with the trigger microparticles and the biotinylated secondary antibodies, to the sample to be assayed. Moreover, biotinylated antibodies may be prepared by rapid and simple procedures well known to those of skill in the art. FIGURE 3 illustrates schematically an assay performed using this method.

In some embodiments, detection microparticles and trigger microparticles may be labeled with fluorophores that have the same excitation wavelengths, but distinct emission wavelengths. Thus, a single wavelength of light can be used to excite both the trigger microparticle and the detection microparticle, but the fluorescent signals produced in response by the trigger and detection microparticles are distinct and may be resolved by the MLSC optics. This allows the MLSC system to perform both the identification and quantitation functions described above using a single excitation wavelength. For example, the MLSC system can use the fluorescent signal from the trigger microparticle to determine the identity of the trigger microparticle by measuring the spatial characteristics of the fluorescent signal, and then quantitate the amount of detection microparticle (and hence soluble factor bound to the trigger microparticle) by measuring the magnitude of the fluorescence signal from the detection microparticle.

In other embodiments, the detection microparticles – and optionally the trigger microparticles also – can be labeled with fluorophores that participate in fluorescence resonance energy transfer (FRET). Preferably, the FRET pairs associated with the detection and trigger microparticles are selected such that the donor fluorophores in each are excited by the same excitation wavelength, but the acceptors emit at different wavelengths. Microparticles (beads) of this type have been described in the art. See United States Patent No. 5,573,909, incorporated herein by reference. Thus, each bead can contain a mixture of two or more fluorescent dyes having overlapping excitation and emission spectra, allowing energy transfer from the excitation wavelength of the first dye (donor), transfer through the dyes in the series and reemitted as a signal at the emission wavelength of the last dye

(acceptor) in the series. Indeed, in preferred embodiments, the FRET mixtures in the detection and trigger beads are selected such that the donor fluorophores in each bead set are excited by the same excitation wavelength. The donor fluorophores could be the same for both bead sets or, alternatively, the fluorophores could have different structures provided they are both excitable by the same wavelength. The final acceptors in each bead emit at sufficiently different wavelengths to allow spectral differentiation. In this way the two types of beads (trigger and detection) can be separately identified and the signal from the detection bead can be separately quantitated. A significant advantage of this approach is that only one laser (one wavelength) is required for the excitation of both trigger and detection bead. Available microparticle technology requires the use of two different lasers (wavelengths): a first laser specific for the dye(s) associated with the bead or other support and a second laser specific for the dye(s) used to detect and quantify the analyte (e.g., the fluorophore attached to detection antibody). As one example, the LabMAP™ technology from Luminex Corp. (Austin, TX) requires the use of both (i) a red laser to excite the two fluorescent dyes within the polystyrene microspheres that allow identification of the microsphere; and (ii) a green laser to excite a third dye on the surface of the microsphere to measure the bioassay.

Another advantage of the present invention is that donor and acceptor particles are in close proximity. Thus energy transfer can occur not only by spectral overlap (Foerster type of energy transfer) but also by electron exchange (Dexter type energy transfer) which occurs when the donor and acceptor molecules are in close enough proximity to one another that their electron clouds overlap. See Dexter, J. Chem. Phys. 21:838 (1953).

Finally, the present invention can take advantage of the "Perrin formulation" which describes an effective "quenching sphere" around the donor molecule. If an acceptor is within this quenching sphere, then energy transfer occurs with very high efficiency. This in turn means that energy transfer can occur without spectral overlap. This may be advantageously exploited in the present invention by selecting donors and acceptors that do not need overlap of the respective emission and excitation spectra.

In other embodiments, the invention provides a system for the detection and quantitation of a soluble factor in which the reagents comprise: (a) a trigger microparticle coupled to a primary reagent capable of binding to a soluble factor; (b) a modifying reagent capable of introducing a ligand group onto said soluble factor; and (c) a dye-labeled detection microparticle coupled to a secondary reagent capable of binding to said ligand group.

In this series of embodiments, biological fluid suspected of containing the soluble factor of interest is treated with a modifying reagent that adds a chemical group that can serve as a ligand for the secondary reagent. For example, a biological fluid suspected of containing proteins as the factors of interest can be treated with a biotinylating reagent, such as EZ Link™ Sulfo-NHS-LC-Biotin (Pierce Chemical Co.). This compound adds a biotin group to any soluble factor that has an amine group, including proteins. Dye-conjugated detection microparticles can then be conjugated with a group that specifically binds to the ligand group. In embodiments where the ligand group is biotin, the detection microparticles can be conjugated with streptavidin. The biological fluid is then treated with trigger microparticles that are labeled with an antibody against the soluble factor of interest. Following an optional wash step to remove excess unconjugated biotin, streptavidin-labeled detection microparticles are added. Biotinylated-soluble factor can bind to both the trigger microparticles (via the interaction with the trigger-microparticle antibody) and the detection microparticles (via the interaction with the detection-microparticle streptavidin group), thereby bringing the detection microparticles together in a complex with the trigger microparticles. The amount of the soluble factor of interest can be determined by measuring the magnitude of the detection-microparticle dye signal associated with each trigger microparticle in the assay. As in the above embodiments, the use of populations of trigger microparticles that bind to different soluble factors and have unique physical properties will permit the multiplexed analysis of many different soluble factors in each assay.

In some preferred embodiments, when the soluble factor of interest is known or can be isolated, the assay may be performed in a competitive assay format. In this embodiment, purified soluble factor of interest is first conjugated to a ligand (e.g., biotin). Then, a predetermined amount of this modified exogenous soluble factor is contacted with the biological fluid to be assayed, along with trigger microparticles labeled with an antibody against the soluble factor of interest, and fluorescently labeled detection microparticles coupled to streptavidin groups. The ligand-modified exogenous soluble factor will compete with the unmodified endogenous soluble factor in the biological fluid for binding to the antibody on the trigger microparticles. The streptavidin groups on the detection microparticles will bind to the biotin groups on the exogenous modified soluble factor and will not bind to the endogenous soluble factor. As a result, the detection microparticle signal will be inversely proportional to the amount of endogenous soluble factor in the biological fluid. This is illustrated in FIGURE 4. By conducting the assay with known concentrations of modified

exogenous soluble factor, the amount of endogenous soluble factor in the biological fluid can be determined. Again, by using multiple populations of trigger microparticles with unique physical characteristics, it is possible to perform multiplexed analysis of soluble factors. It will be appreciated that other embodiments of the invention can be adapted to take advantage of various competitive assay formats. For example, the assay can be performed by first conjugating the ligand (e.g., biotin) to the endogenous soluble factor in the biological fluid, then adding unmodified exogenous soluble factor. In this scheme, the greater the amount of endogenous soluble factor, the great the detection microparticle signal.

In the assays described above, biotin is the preferred ligand group introduced by modification; however, other ligand groups are contemplated by the invention (e.g., DNA and RNA oligonucleotides). Detection microparticles conjugated to complementary nucleic acid molecules can bind to these ligands to facilitate detection of the soluble factor.

Known assays for soluble factors commonly use a primary antibody coupled to a solid support (e.g., a bead) to capture the soluble factor. The amount of soluble factor that has been captured is determined by introducing a dye-labeled secondary antibody against the soluble factor. In the present invention, the dye signal used to quantify the soluble factor is not associated with a soluble secondary reagent, but is instead associated with a detection microparticle. This results in a substantial advantage over prior art systems because the detection microparticles can be labeled with significantly more dye than a single secondary antibody. As a result, the binding between a trigger microparticle bound factor and a detection microparticle contributes significantly more dye signal than a single secondary antibody to the factor. The higher dye signal means that the system provided herein is several orders of magnitude more sensitive than prior art systems using dye-labeled secondary antibodies alone. This increased sensitivity can be exploited to allow the system to acquire data at substantially higher rates than prior art systems, thereby lending itself to high-throughput applications. In addition, the increased sensitivity can be exploited to allow the system to detect factors that are present at low levels in the sample.

Detection microparticles also can be labeled with combinations of dyes in the same way as the trigger microparticles as described above. By this procedure, it is possible to increase even further the power of the assay, as each soluble factor will have a unique combination of detection microparticle signal (size and/or fluorescent signal, for example) and trigger microparticle signal. It will be appreciated by those skilled in the art that the present invention allows for the use of combinations of detectable characteristics for the performance

of multiplexed assays. As a result, the present invention allows assays to be performed that are able to accommodate considerably more complexity than prior art systems for multiplexed assays.

In preferred embodiments of the invention, the trigger microparticle is labeled with a high density of primary reagent, thereby maximizing the sensitivity of the assay for the soluble factor. In some preferred embodiments, the detection microparticles are coupled to only a limited number of "copies" of the secondary reagent; in some applications, even one "copy" per microparticle may be preferred. In this way, a maximum number of detection microparticles can bind to an individual trigger microparticle. This localized concentration of the detection microparticles on the trigger microparticle generates a high dye signal above the background generated by the unbound detection microparticles.

In other embodiments, the detection microparticle has multiple copies of the secondary reagent on its surface. When the secondary reagent is an antibody, crosslinking or agglutination of trigger microparticles and detection microparticles can occur in which one or more trigger microparticles bind to one or more detection microparticles. Agglutination immunoassays are well-known in the art and are often based on crosslinking polystyrene microparticles, where agglutination is detected by light scattering measurements. The methods of the present invention allow agglutination assays that are more sophisticated than such prior art assays. For example, the present invention allows competitive mixed microparticle agglutination assays. Although this type of assay is best suited for small soluble factors, such as hormones and drugs, it can also be useful for larger molecules. In the assay, the soluble factor to be measured (D) is coupled to a dye-conjugated microparticle by means known in the art (e.g., via linker or an antibody). A high affinity antibody that recognizes factor D is coupled to a second dye-conjugated microparticle; preferably, different dyes are used for the two types of microparticles. Both microparticle types are mixed together with the sample to be assayed. In the absence of free factor D in the sample, agglutination will occur as the anti-factor D antibodies on the first type of particle will bind to the factor D bound to the second type of particle. The agglutination and will be revealed by MLSC as patches of mixed fluorescence. In the presence of free factor D in the sample, however, agglutination will be inhibited or reduced as a function of the concentration of free factor D. This is because the anti-factor D antibodies on the first type of particle will bind to the free factor D in the sample. Accordingly, the degree of agglutination inhibition can be used to quantify the amount of free factor D in the sample. This process is illustrated in FIGURE 5. In addition, the ability of the

flow cytometer system to discriminate between many different types of microparticles based on their attributes (e.g., size, color, fluorescence profile, etc.) allows such agglutination and competitive assays to be multiplexed.

Another series of embodiments contemplated by the present invention include non-competitive microparticle agglutination assays. Such assays are possible when the soluble factor to be quantitated has two or more non-overlapping epitopes. For example, if the soluble factor has non-overlapping epitopes, A and B, then two sets of microparticles could be prepared: One set of microparticles (labeled with a dye) and be coupled to anti-A antibodies while a second set of microparticles (labeled with a different dye) would be coupled to anti-B antibodies. In the presence of the soluble factor of interest, mixed agglutinates will be formed as shown in FIGURE 6. These mixed agglutinates would be detected by flow cytometry (e.g., MLSC). In the absence of the soluble factor the mixed agglutinates would not be formed. As with the previous example, described above, the degree of agglutination will be proportional to the concentration of soluble factor. The two-site recognition of mixed agglutination improves the specificity of this assay and largely avoids nonspecific particle agglutination and its associated problems. Furthermore, the ability of the flow cytometer to discriminate between many different types of microparticles based on their attributes will also allow such mixed agglutination and competitive assays to be multiplexed.

The embodiments described above illustrate the methods of the present invention for the detection of soluble factors contained in samples using antibodies as the primary reagents. The methods of the invention are also applicable to other types of assays for which multiplexing is desirable. For example, the methods of the invention can be applied to analyze nucleic acid obtained from a subject. The nucleic acid can either be used directly in a sample, or isolated from a sample and amplified, for example by the polymerase chain reaction (PCR). In one embodiment, PCR is performed on DNA obtained from a subject using primers to amplify specifically regions of the genome suspected of harboring mutations. Synthetic oligonucleotides that bear the mutations to be identified are coupled to trigger microparticles, such that each population of trigger microparticles is coupled to a synthetic oligonucleotide specific for a particular mutation. Each population of trigger microparticles is then contacted with the amplified nucleic acid from the sample and also with biotinylated oligonucleotides with a sequence complementary to the one present on the surface of the trigger microparticles. If the subject is "wild-type" for the particular mutation (i.e., the mutation is absent), then amplified DNA from the sample will not hybridize with the oligonucleotides on the trigger

microparticles (or to the biotinylated oligonucleotides). As a result, the trigger microparticle oligonucleotide will hybridize with the biotinylated oligonucleotide.

If streptavidin-labeled fluorescent detection microparticles are then added to the assay, this will lead to complexes of trigger microparticles and detection microparticles. The level of binding between the microparticles can be quantitated as described above; however, if the subject has a mutation, the amplified DNA from the sample will hybridize to the biotinylated oligonucleotide, thus preventing this biotinylated oligonucleotide from hybridizing to the oligonucleotide coupled to the trigger microparticle. The addition of streptavidin-labeled fluorescent detection microparticles will not lead to the formation of complexes of trigger microparticles and the detection microparticle. Thus, relative to a subject with a wild-type sequence, the detection microparticle fluorescence associated with the trigger microparticle will be reduced in the presence of the mutation. In this way, the level of detection microparticle binding to trigger microparticle can reveal the presence or absence of the particular mutation that the trigger microparticle is designed to detect. In multiplexed assays, each mutation is assayed using a separate trigger microparticle and biotinylated oligonucleotide pair. Thus, the present invention encompasses a method for rapidly performing mapping and analysis of single nucleotide polymorphisms (SNPs).

In other embodiments, the assays can be performed using an oligonucleotide bearing the wild-type sequence that is bound to the trigger microparticle, and a biotinylated oligonucleotide with a complementary sequence. In this embodiment, the presence of the mutation in the assay sample will not impede the hybridization of the biotinylated oligonucleotide to complementary oligonucleotide associated with the trigger microparticle. Thus, when the mutation is present, the fluorescent detection microparticles (conjugated to streptavidin) will bind to the trigger microparticles. Accordingly, the presence of the mutation in the subject will result in greater detection microparticle signal at the trigger microparticle than if the individual is wild-type. Thus, it will be clear to those skilled in the art that the present invention will allow the rapid, simultaneous determination of genotypic information.

In other embodiments of multiplexed analysis of nucleic acid sequences, populations of trigger microparticles are prepared with all possible variants of the sequence of interest. The test sample is amplified (by PCR or otherwise) using upstream primers modified with biotin at the 5' end. After denaturation, the amplified nucleic acid is contacted with the trigger microparticles under hybridization conditions. Addition of streptavidin-labeled fluorescent detection microparticles will identify the sequence of the PCR products. Thus wild-type,

known mutants and unknown mutants can be identified and quantitated simultaneously. Using the methods of the present invention, genetic variations such as point mutations, deletions or insertions could be determined in a very efficient manner.

Alternatively, or in combination, the methods of the present invention can be used to detect and identify pathogen(s) that are suspected of infecting the individual. In one embodiment of the pathogen assay, the trigger microparticle bears an oligonucleotide complementary to a region of the pathogen genome, while the detection microparticle bears the complementary sequence. The presence of pathogen DNA in the assay sample is revealed if the detection microparticle signal associated with the trigger microparticle is lower in the presence of sample than in the absence, because any pathogen DNA in the sample will competitively inhibit the hybridization between the detection and trigger microparticle. In the case of RNA-bearing pathogens, complementary DNA may be amplified for the assay. In another embodiment, trigger microparticles can be prepared that are labeled with oligonucleotides specific for pathogens. As described above the derivatized particles can capture biotinylated pathogenic amplified nucleic acid that in turn will be detected by addition of streptavidin-labeled fluorescent detection microparticles.

It is to be understood that all of the assays described above can be combined to yield a multiplexed assay that simultaneously assays such diverse biological properties as genotype at a particular locus, presence of pathogens, and soluble factor concentration.

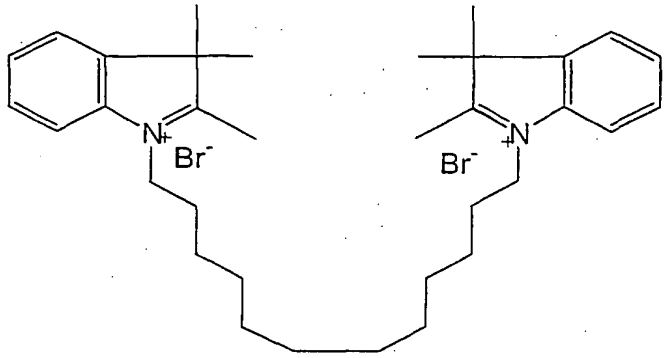
EXAMPLES

The following examples are provided to allow those skilled in the art access to information regarding various embodiments of the present invention, and are not intended in any way to limit the scope of the invention.

EXAMPLE 1

Synthesis of dyes suitable for incorporation into trigger and detection beads

A. Synthesis of C12 linked 2.3.3- trimethyl-indolenine (compound #1).

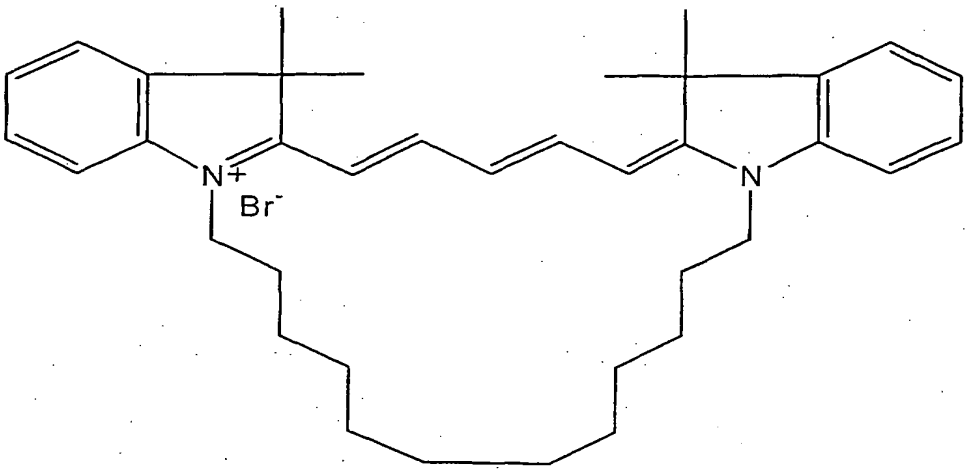


Dibromododecane (4.3 g, 13.3 mmol) was added to neat 2,3,3-trimethyl indolenine (8.5 g, 54.3 mmol). The solution was heated to reflux and stirred for 24 h. The solution was cooled to room temperature and the crude reaction material was loaded onto a silica gel column and chromatographed (0 to 20% MeOH/CH_2Cl_2). The desired fractions of product, 1, were collected and concentrated to yield a hygroscopic purple glassy solid. Yield- 7.0 g (79%)

R_f = 0.5 (5% MeOH/CH_2Cl_2).

^{1}H NMR ($CDCl_3$, 360 MHz) δ 7.57 (m, 8H), 4.75 (t, 4H, J= 7.4 Hz), 3.12 (s, 6H), 1.95 (m, 4H), 1.51 (s, 12H), 1.26-1.45 (m, 16H).

B. Synthesis of Dodecanyl Bridged Indoleninium Pentamethine Dye (compound #2)



Compound #1 (66 mg, 0.10 mmol) was added and dissolved in acetic anhydride (3 ml). To that solution, sodium acetate (500 mg) and malonaldehyde phenyl imine (28 mg, 0.10 mmol) were added respectively. The solution was heated to 135°C for 2 hours. The reaction was

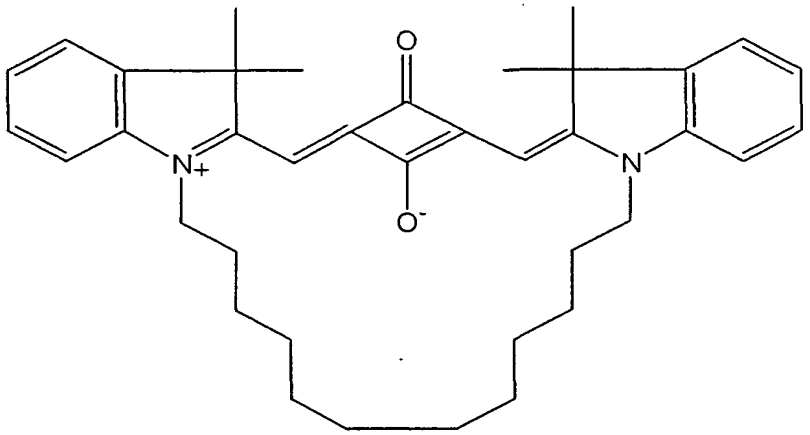
monitored spectrophotometrically by the appearance of a peak at 656 nm (CH_2Cl_2). The solution was cooled to room temperature and the crude solution was concentrated on a rotovapor. The concentrate was then loaded onto preparatory TLC plates (silica, 10% MeOH in CH_2Cl_2) to obtain the desired purified product 11. Yield 50 mg (81%) R_f= 0.3 (5% MeOH in CH_2Cl_2).

λ_{max} (CH_2Cl_2) 656 nm, λ_{em} (CH_2Cl_2) 674 nm.

^{1}H NMR ($CDCl_3$, 360 MHz) δ 8.20 (m, 2H), 7.65 (d, 2H , J=7.4 Hz), 7.15-7.38 (m, 5H), 7.05 (dd, 2H, J=1.2 Hz, 7.4 Hz), 6.22 (m,2H), 4.04 (bt, 4H), 1.86 (m, 4H), 1.20-1.50 (m, 28H).

MS (FAB, NBA), calculated for $C_{37}H_{49}N_2Br$, 601, found 521 (M-Br, 15%).

C. Synthesis of Dodecanyl Bridged Indoleninium squaraine Dye (compound #3)

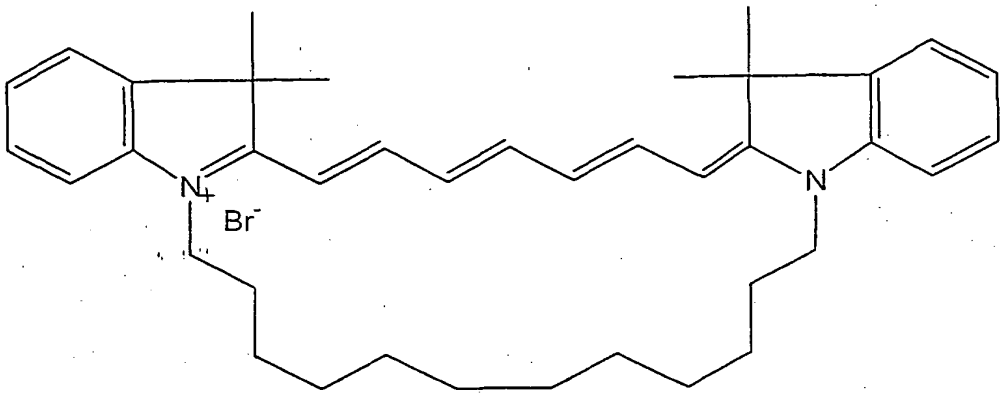


Compound #1, (66 mg, 0.10 mmol) was dissolved in 1:1 (v/v) n-butanol and toluene (3 ml). To that solution, 3,4 di-hydroxy-3cyclobuten-1,2-dione (11 mg, 0.10 mmol) was added. The solution was heated to 135°C for 2 hours. The solution was cooled to room temperature and 5 ml of water was added. The solution was then extracted three times with 15 ml of dichloromethane. The extract was dried with sodium sulfate, filtered using vacuum filtration, and concentrated on a rotovapor. The crude concentrate was purified using preparative TLC (2% MeOH in CH_2Cl_2) to obtain the desired purified product compound #3. Yield 30 mg (50%) R_f = 0.35 (5% MeOH in CH_2Cl_2).

λ_{max} (CH_2Cl_2) 639 nm, λ_{em} (CH_2Cl_2) 648 nm.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.32 (m, 4H), 7.13 (bt, 2H), 6.96 (bt, 2H), 5.95 (s, 2H), 3.99 (bt, 4H), 1.15-1.45 (m, 32H).

D. Synthesis of Dodecanyl Bridged Indoleninium Heptamethine Cyanine Dye (compound #4)



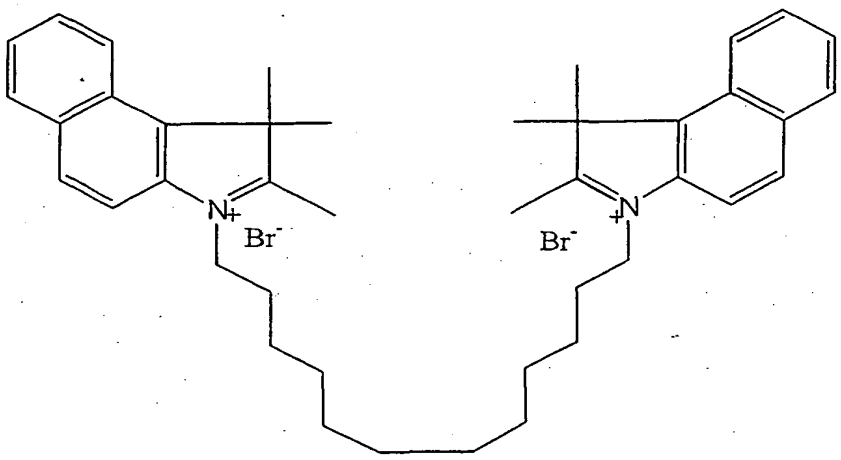
Compound #1, (400 mg, 0.61 mmol) was added and dissolved in acetic anhydride (4 ml). To that solution, sodium acetate (690 mg) and glutaconic aldehyde phenyl imine (195 mg, 0.68 mmol) was added respectively. The solution was heated to 135°C for 2 hours. The solution was cooled to room temperature and the crude solution was concentrated on a rotovapor. The concentrate was then loaded onto preparatory TLC plates (10% methanol/ dichloromethane) to obtain the desired purified product compound #4. Yield 190 mg (49%) $R_f = 0.6$ (5% MeOH in CH_2Cl_2).

λ_{max} (CH_2Cl_2) 760 nm, λ_{em} (CH_2Cl_2) 790 nm.

^1H NMR ($CDCl_3$, 360 MHz) δ 7.78 (m, 2H), 7.45-7.65 (m, 4H), 7.47 (m, 3H), 7.00-7.21 (m, 4H), 6.24 (bd, 2H).

MS (FAB, NBA), calculated for $C_{39}H_{51}N_2Br$ 627, found 547 (M-Br, 60%).

E. Synthesis of C12 linked 2,3,3-trimethyl-benzindolenine (compound #5)

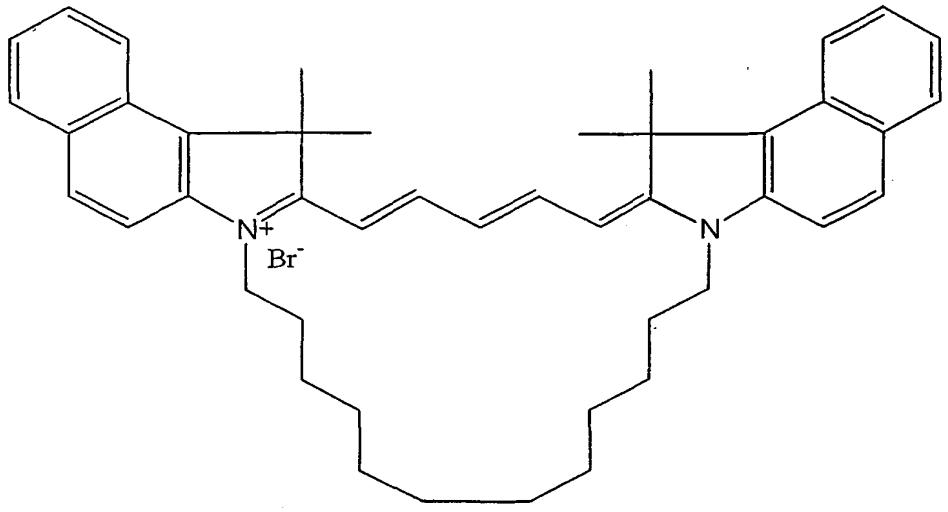


Dibromododecane (1.9 g, 5.9 mmol) was dissolved in *o*-dichlorobenzene (5 ml). 2,3,3-trimethylbenzindolenine (5.0 g, 23.8 mmol) was added and the solution was heated to reflux

for 24 hours. The reaction was cooled to room temperature and the crude solution was loaded onto a silica gel column and chromatographed (0 to 20% MeOH in CH_2Cl_2) to obtain the desired product compound #5. Yield 3.0 g (66%) $R_f = 0.6$ (5% MeOH in CH_2Cl_2).

^1H NMR ($CDCl_3$, 360 MHz) δ 8.06 (d, 2H), 8.02 (d, 2H) 7.97 (d, 2H), 7.79 (d, 2H), 7.66 (dd, 2H), 7.60 (dd, 2H), 4.78 (t, 4H), 3.17 (s, 6H), 1.95 (m, 4H), 1.80 (s, 12H), 1.46 (m, 4H), 1.40 (m, 4H), 1.19 (m, 8H).

F. Synthesis of Dodecanyl Bridged Benzindoleninium Pentamethine Cyanine Dye (compound #6)

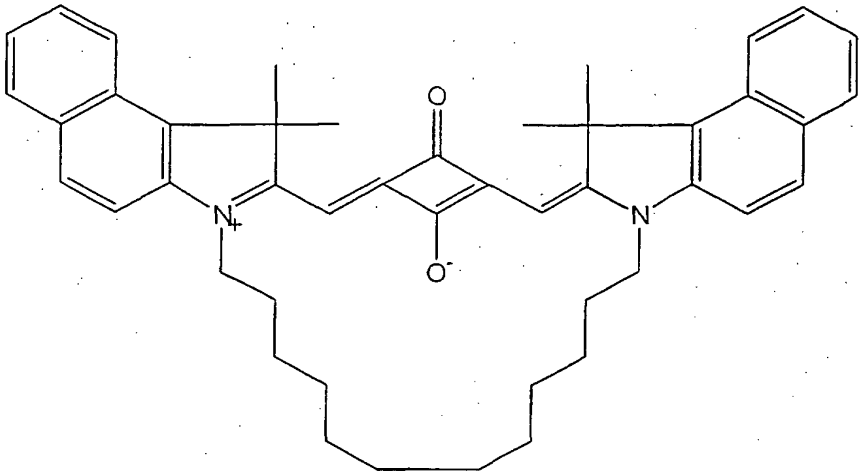


Compound #5 (415 mg, 0.55 mmol) was added and dissolved in acetic anhydride (3 ml). To that solution, sodium acetate (500 mg) and malonaldehyde phenyl imine (142 mg, 0.55 mmol) was added respectively. The solution was heated to 135°C for 2 hours. The solution was cooled to room temperature and the crude solution was concentrated on a rotovapor. The concentrate was then loaded onto preparatory TLC plates (10% methanol/ dichloromethane) to obtain the desired purified product compound #6. Yield 210 mg (52%) $R_f = 0.6$ (5% methanol/dichloromethane).

λ_{max} (CH_2Cl_2) 696 nm, λ_{em} (CH_2Cl_2) 716 nm.

^1H NMR ($CDCl_3$, 300 MHz) δ 8.17 (m, 2H), 7.78 (m, 2H), 7.72 (t, 1H, J= 14 Hz), 7.53 (d, 4H, J = 8.4 Hz), 7.42 (m, 2H), 7.28 (d, 2H, J= 8.4 Hz), 7.14 (t, 2H, J= 7.6 Hz), 6.24 (d, ~2H, J= 14 Hz).

G. Synthesis of Dodecanyl Bridged Benzindoleninium squaraine Dye (compound #7)



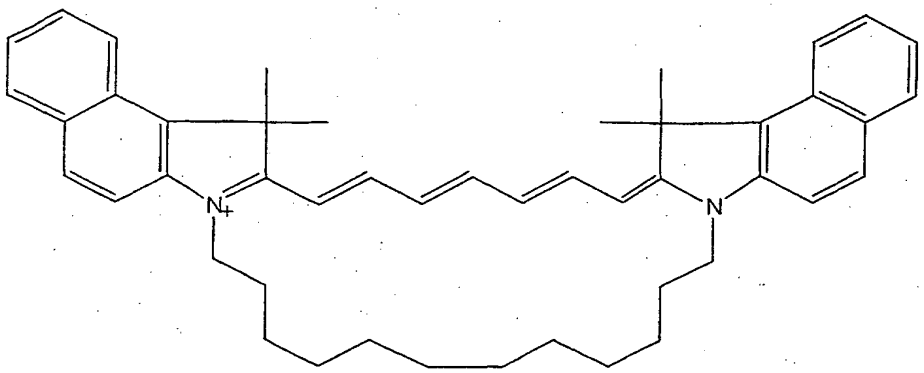
Compound #5, (375 mg, 0.49 mmol) was added and dissolved in 1:1 (v/v) n-butanol and toluene (3 ml). To that solution, 3,4 hydroxy-3-cyclobuten-1,2-dione (55 mg, 0.49 mmol) was added. The solution was heated to 135°C for 2 hours. The solution was cooled to room temperature and 5 ml of water was added. The solution was then extracted three times with 15 ml of dichloromethane. The extract was dried with sodium sulfate, filtered using vacuum filtration, and concentrated on a rotovapor. The crude concentrate was purified using preparatory TLC (2% MeOH in CH_2Cl_2) to obtain the desired purified product compound #7, Yield 150 mg (45%) $R_f = 0.7$ (5% MeOH in CH_2Cl_2).

λ_{max} (CH_2Cl_2) 671 nm, λ_{em} (CH_2Cl_2) 681 nm.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.10 (d, 2H, J= 8.4 Hz), 7.83 (m, 4H), 7.53 (m, 2H), 7.42 (d, 2H, 8.4 Hz), 7.31 (m, 2H), 6.05 (bs, 2H), 4.12 (bt, 4H), 1.88 (m,4H), 1.17-1.62 (m, 28H)

MS (FAB, NBA) calculated for $C_{46}H_{50}N_2O_2$ 662, found 663 ($M+H^+$, 45%).

H. Synthesis of Dodecanyl Bridged Benzindoleninium Heptamethine Dye (compound #8)



Compound #5, (256 mg, 0.34 mmol) was dissolved in acetic anhydride (4 ml). To that solution, sodium acetate (600 mg) and glutaconic aldehyde phenyl imine (95 mg, 0.34 mmol)

was added respectively. The solution was heated to 135°C for 2 hours. The solution was cooled to room temperature and the crude solution was concentrated on a rotovapor. The concentrate was then loaded onto preparatory TLC plates (10% methanol/ dichloromethane) to obtain the desired purified product compound #8. Yield 120 mg (50%) $R_f = 0.6$ (5% MeOH in CH_2Cl_2).

λ_{max} (CH_2Cl_2) 800 nm, λ_{em} (CH_2Cl_2) 820 nm.

^1H NMR ($CDCl_3$, 300 MHz), δ 8.10 (m, 2H), 7.80-7.92 (m, 6H), 7.24-7.60 (m, 7H), 6.60 (m, 2H), 6.21 (m, 2H), 4.10 (bt, 4H), 1.87 (m, 4H), 1.15-1.48 (m, 28H).

EXAMPLE 2

Dyeing of 9.7 μm Beads

Beads of diameter 9.7 μm are suitable for use as trigger beads in MLSC assays for soluble factors. A bead solution, containing 5 mg of beads (200 μl), was placed in a vial and washed to remove excess detergent. The solution was washed by centrifuging the solution at approximately 2000 rpm and decanting the supernatant. The remaining beads were suspended in 800 μl of water. To the bead solution, 20 μl of a 0.4 μm dye solution compound #2 was added. The solution was then agitated for 24 hours. The solution was then centrifuged at 2000 rpm and the supernatant was decanted to remove excess dye. The remaining beads were suspended in PBS (phosphate buffered saline)/ 0.1% TWEEN® solution. The washing process was repeated two more times. The fluorescent intensity of the beads was then evaluated on the Surroscan instrument.

EXAMPLE 3

Physical Adsorption of Antibody onto Dyed 9.7 μm Beads

An aliquot of 9.7 μm compound #5 dyed bead solution, containing 10 mg of beads (833 μl), was placed in a vial and washed three times. The beads were washed by adding 1 ml of 10 mM phosphate buffer (pH 8), centrifuging at 10,000 rpm, and decanting supernatant. The final portion of beads was then suspended in 100 μl of phosphate buffer. An antibody solution, containing 200 μg (50 μl) of antibody (in this case, the anti-human Ig antibody obtained from PharMingen), was diluted to 2 ml with phosphate buffer in a glass vial. The bead solution was then added to the antibody solution. The solution was agitated at 4°C overnight. The solution was then transferred to a Falcon® tube and 4 ml of a PBS/1% BSA (Bovine Serum Albumin) solution was added. The solution was centrifuged at 10,000 rpm and the supernatant decanted. The remaining beads were then suspended in 4 ml of PBS/BSA

solution. The washing process was repeated three more times. The final quantity of beads was diluted to 8 ml with PBS/BSA.

EXAMPLE 4

Dyeing of 40 nm Beads

Beads of diameter 40 nm are suitable for use as detection beads in the assays of the present invention. A bead solution, containing 20 mg of beads (400 μl), was placed in a vial and diluted to 1 ml with water. The solution was placed in a sand bath preheated to 70°C and was allowed to equilibrate. 50 μl of a 10 mM compound #4, dye solution was then added to the bead solution dropwise at the rate of 2 drops every 2 minutes. The solution was then allowed to slowly cool to room temperature and then allowed to stir overnight. The beads solution was then filtered using a tangential flow filtration device. The beads were filtered through with water. The final solution of beads was then collected from the device and stored in a Falcon® tube.

EXAMPLE 5

Procedure for Biotinylation of Protein or Amine-Containing Soluble Factors

Protein (in this example, human IgG obtained from Kirkegard & Perrin Laboratories) dissolved in PBS buffer pH 7.4 (1 ml at 1 mg/ml) was treated with a solution of EZ Link™ Sulfo-NHS-LC-Biotin (Pierce) (10 μl at 10 mg/ml in DMSO) for 2 hours at room temperature. The mixture was then dialyzed against PBS buffer (3x2L). The conjugate was used without further purification.

EXAMPLE 6

Coating of 40 nm Dyed Beads with Streptavidin

A solution of compound #4 dyed particles (40 nm) was constituted to 20 mg/ml in MES buffer (pH 6.0, 10 mM). To a 1.0 ml solution was added 10 μl of a freshly prepared solution of EDAC (10 mg/ml) in water. The reaction mixture was incubated in an end-over-end shaker for 15 minutes. The activated bead suspension was then added to a streptavidin solution (100 μl of 0.5 mg/ml) and the pH adjusted to 7.5 with phosphate buffer. The coupling reaction was allowed to proceed for 2h and then quenched with 1 ml of 1M glycine (pH 9.0). The particles were then purified by dialysis (Spectrum, CA) or tangential diafiltration (400K hollow fibers from Microgon, Laguna Hills CA).

EXAMPLE 7

General Procedure for Running Assay

Approximately 12,000, 9.7 μm dyed beads physically absorbed with antibody in 5 μl of diluent (PBS/BSA/EDTA/Azide) is mixed with the soluble factor which is dissolved in 5 μl of diluent for approximately 30minutes. During this time, approximately 10^9, 40 nm beads in 2.5 μl of water is mixed with approximately 10^{10} molecules of biotin coated antibodies in 2.5 μl of water. The bead solutions are then mixed together and agitated for 24 hrs. The entire solution is then used for evaluation on the Surroscan. The assay solution is injected into a Flex32 capillary plate. The capillary holds a volume of approximately 5 μl, so approximately 4000 particles of 9.7 μm beads will be in the capillary for evaluation. The Surroscan then evaluates these beads and the data is translated into files using Flowjo analysis software. In Flowjo, random or non specific events can be filtered out from those events which are of interest. The events can then be analyzed for their fluorescent intensity which can be used to evaluate the levels of soluble analyte in the assay.

CLAIMS

We claim:

1. A method for the simultaneous quantitation of a plurality of soluble factors in a clinical sample using flow cytometry, the method comprising:

(a) providing populations of trigger microparticles, each said population coupled to a primary reagent capable of specifically binding to one of said soluble factors such that each population of trigger microparticles binds to a different soluble factor than the remaining populations of trigger microparticles, and wherein each said population of trigger microparticles can be distinguished by flow cytometry;

(b) providing secondary reagents, each said secondary reagent capable of binding to one of said soluble factors;

(c) providing detection microparticles, said detection microparticles capable of binding specifically to said secondary reagents, and said detection microparticles associated with a detection dye;

(d) contacting said clinical sample with said populations of trigger microparticles, said secondary reagents, and said detection microparticles;

(e) identifying each said trigger microparticle in said clinical sample using flow cytometry;

(f) quantitating using flow cytometry the amount of each said soluble factor in said clinical sample by measuring the detection dye signal associated with each said trigger microparticle identified in step (e).

2. The method of claim 1 wherein each said population of trigger microparticles is coupled to a different identification dye, each said identification dye having a unique spectral profile different from said detection dye, and wherein the unique physical characteristic of each said population of trigger microparticles comprises the unique spectral profile from said identification dye.

3. The method of claim 2 wherein said identification dyes and said detection dyes are fluorophores.

4. The method of claim 2 wherein said identification dye is a phosphorescent molecule.

5. The method of claim 2 wherein said detection dye is a phosphorescent molecule.

6. The method of claim 1 wherein each said population of trigger microparticles is coupled to at least two different identification dyes different from said detection dye, wherein the ratio of the amount of the identification dyes coupled to each population of trigger microparticles is unique such that each population of trigger microparticles has a unique spectral profile, and wherein said unique physical characteristic of each said population of trigger microparticles comprises the unique spectral profile of said identification dyes.

7. The method of claim 6 wherein said identification dyes and said detection dyes are fluorophores.

8. The method of claim 1 wherein said unique physical characteristic is selected from the group consisting of unique size, unique fluorescence emission intensity, unique fluorescence emission spectrum, unique time-resolved fluorescence emission spectrum, unique phosphorescence profile, unique Raman spectrum, unique pattern of reflectivity, and unique shape.

9. The method of claim 1 wherein said unique physical characteristic is a combination of two or more variables selected from the group consisting of size, fluorescence emission intensity, fluorescence emission spectrum, time-resolved fluorescence emission spectrum, phosphorescence profile, Raman spectrum, reflectivity pattern, and shape.

10. The method of claim 1 wherein said secondary reagent is labeled with biotin and said detection microparticles are labeled with streptavidin, and wherein the specific binding of said detection microparticles to said secondary reagents results from the interaction between biotin and streptavidin.

11. The method of claim 1 wherein said primary and said secondary reagents are antibodies.

12. A method for the simultaneous quantitation of a plurality of soluble factor biological markers in a clinical sample using microvolume laser scanning cytometry (MLSC), the method comprising:

(a) providing populations of trigger microparticles, each said population coupled to a primary reagent capable of specifically binding to one of said soluble factors such that each population of trigger microparticles binds to a different soluble factor than the remaining populations of trigger microparticles, and wherein each said population of trigger microparticles has a unique physical characteristic detectable by MLSC;

(b) providing a modified version of each said soluble factor, said modification comprising the addition of a ligand group;

(c) providing detection microparticles, said detection microparticles coupled to a secondary reagent capable of specifically binding to said ligand group; and wherein each said detection microparticle is coupled to multiple copies of a detection dye;

(d) contacting said clinical sample with said populations of trigger microparticles, said detection microparticles, and said modified versions of said soluble factors; whereby said modified versions of said soluble factors compete with said soluble factors in said clinical sample for binding to said trigger microparticles;

(e) Identifying each said trigger microparticle in said clinical sample by measuring the unique physical characteristics of said trigger microparticles using MLSC;

(f) quantitating using flow cytometry the amount of each said soluble factor in said clinical sample by quantitating the detection dye signal associated with each said trigger bead identified in step (d).

13. The method of claim 12 wherein said ligand group is biotin, and said secondary reagent is streptavidin.

14. A method for the simultaneous quantitation of a plurality of soluble factor biological markers in a clinical sample using microvolume laser scanning cytometry (MLSC), the method comprising:

(a) providing populations of trigger microparticles, each said population coupled to a primary reagent capable of specifically binding to one of said soluble factors such that each population of trigger microparticles binds to a different soluble factor than the remaining populations of trigger microparticles, and wherein each said population of trigger microparticles has a unique physical characteristic detectable by MLSC;

(b) providing populations of detection microparticles, each said population of detection microparticles coupled to a secondary reagent capable of specifically binding to one of said soluble factors at a site distinct from said primary reagent such that each said population of detection microparticles binds to a different soluble factor than the remaining populations, and wherein each detection microparticle is coupled to multiple copies of a detection fluorophore;

(c) contacting said clinical sample with said populations of trigger and detection microparticles;

(d) identifying each said trigger microparticle in said clinical sample by measuring the unique physical characteristics of said trigger microparticles using MLSC;

(e) quantitating using MLSC the amount of each said soluble factor in said clinical sample by quantitating the detection fluorophore signal associated with each said trigger bead identified in step (d).

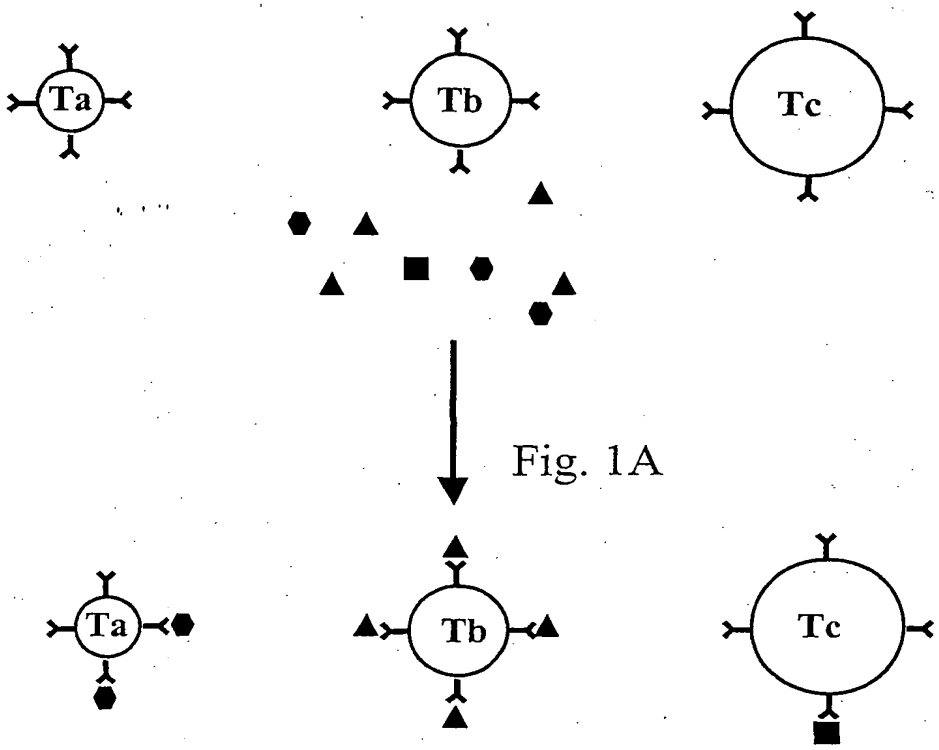


Fig. 1A

Fig. 1B

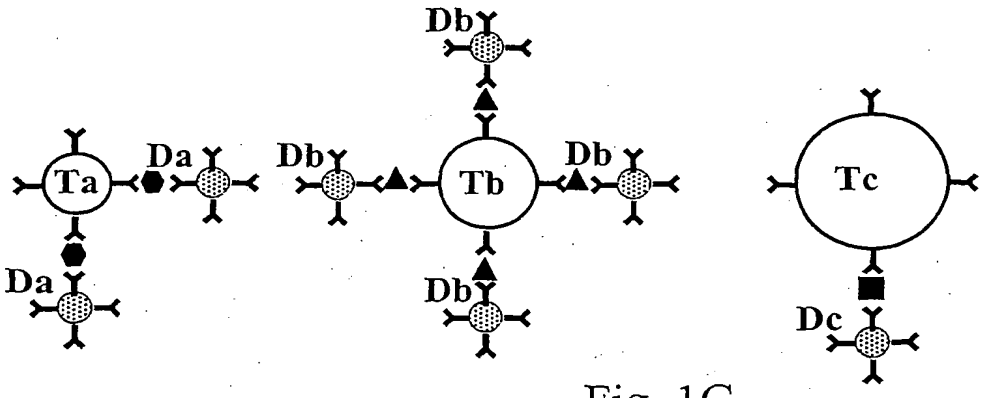


Fig. 1C

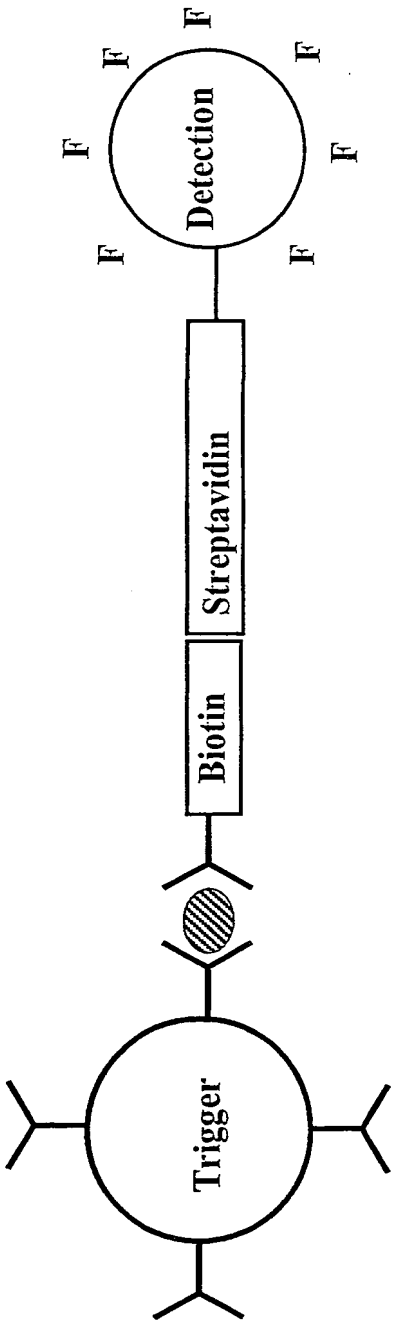


Fig. 2

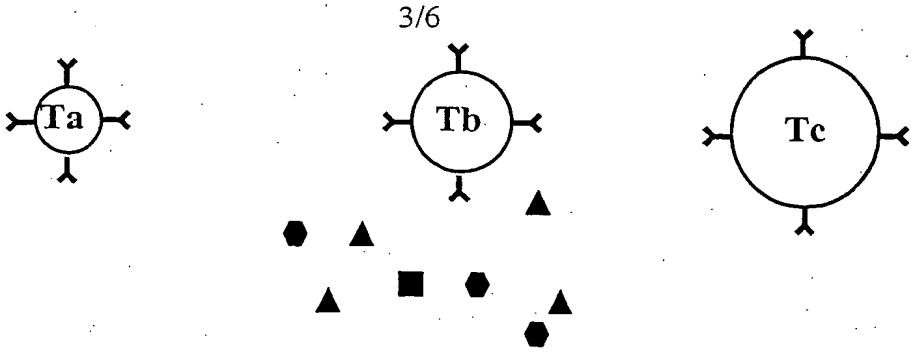


Fig. 3A

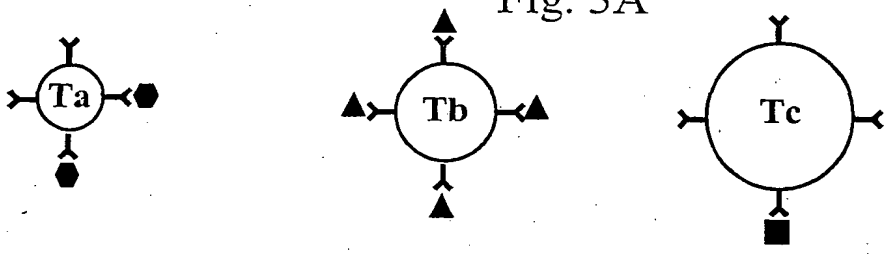


Fig. 3B

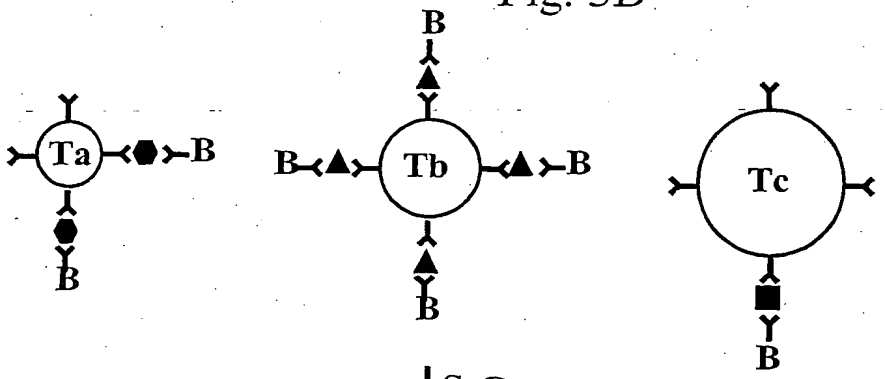


Fig. 3C

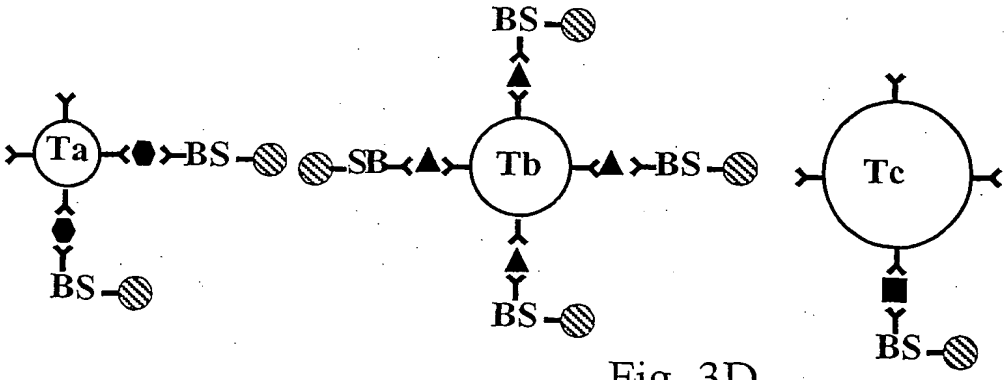


Fig. 3D

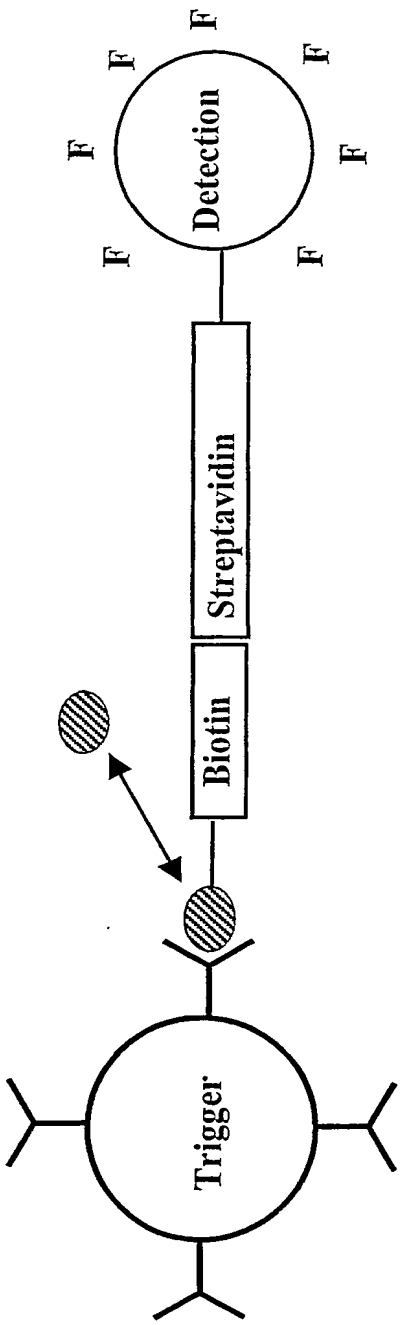


Fig. 4

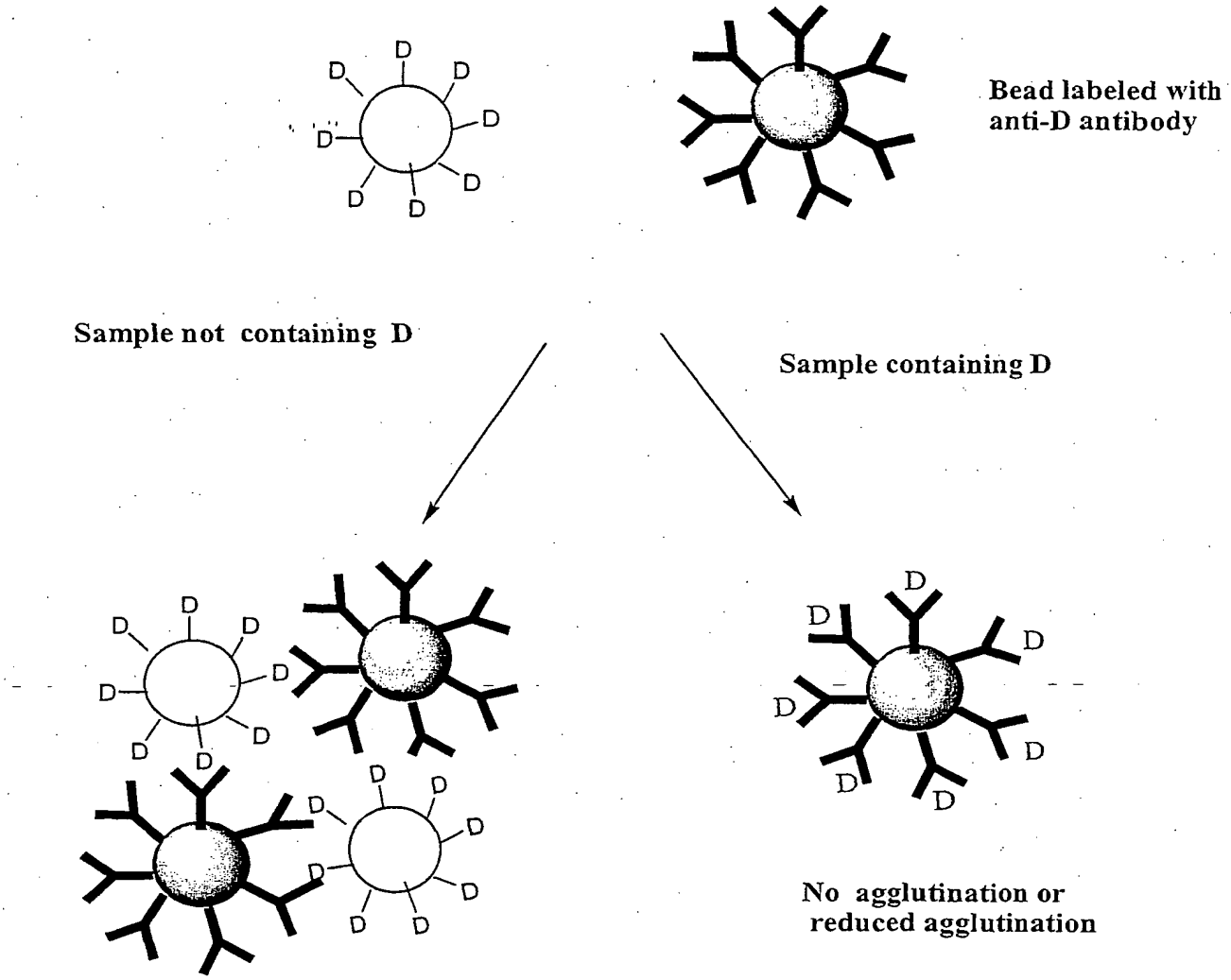


Fig. 5

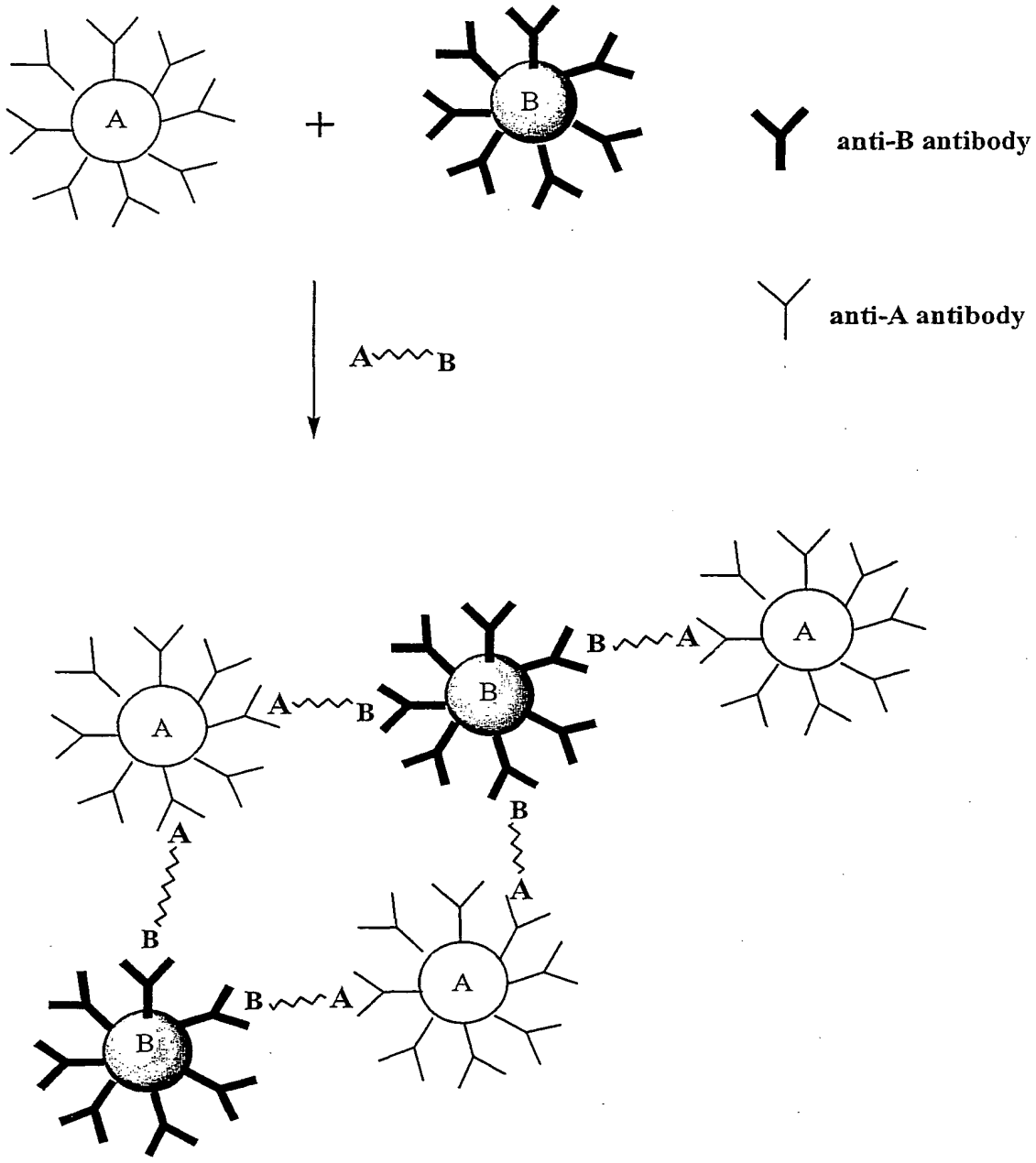


Fig. 6

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(54) Title: ANALYSIS OF SOLUBLE FACTOR BIOLOGICAL MARKERS

(57) Abstract: Multiplexed bioassays of soluble factors by flow cytometry and associated methods and reagents are provided, including the use of trigger microparticles and detection microparticles to form complexes with soluble factors, and methods of detecting and quantitating the complexes in flow cytometer-based, multiplexed assays.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/43583

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : G01N 33/543
US CL : 436/518
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 436/518

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,981,180 A (CHANDLER et al) 09 November 1999 (09.19.1999), column 3, line 58 - column 4, line 67.	1-14
Y,E	US 6,352,862 B1 (DAVIS et al) 05 March 2002 (05.03.2002), column 2, line 50 - column 3, line 4.	1-14
Y	FULTON et al. Advanced Multiplexed analysis with the FlowMetrix TM system. Clinical Chemistry. 19 June 1997 (19.06.1997), Vol. 43, No. 9, column 2, line 1, page 1749 - column 1, line 2 page 1750.	2,3,6-9,11
Y	US 5,891,656 A (ZARLING et al) 06 April 1999 (06.04.1999), column 22, line 60 - column 23, line 34.	4,5
Y,E	US 6,337,184 B1 (MILLER et al) 08 January 2002 (08.01.2002), column 8, lines 34-67.	10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search
15 April 2002 (15.04.2002)

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